STUDIES ON PLASTRON RESPIRATION

IV. PLASTRON RESPIRATION IN THE COLEOPTERA

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(With Plate 7 and Twenty-three Text-figures)

1. INTRODUCTION

The physical and physiological principles of plastron respiration, with special reference to the aquatic Hemipteron *Aphelocheirus*, have already been described in the first three papers of this series (Thorpe & Crisp, 1947*a*, *b*, *c*), while a more general account of the principles involved has also been given (Crisp & Thorpe, 1948). It now remains to consider the structure, life history and physiology of such other insects as are known or suspected to practise this mode of respiration. So far as we know, all other important examples are restricted to the Coleoptera. There are two groups in which it is quite clearly established: the Elmidae of the sub-family Elminae, and the Donaciine (Chrysomelid) genus *Haemonia* (*Macroplea*). Plastron respiration has also been suspected in a third group comprising the weevils of the genus *Phytobius* (Brocher, 1912*c*).

It is the object of the present paper to describe some original experiments on certain members of these groups which throw some light on their respiratory equipment and performance in relation to the general principles which we have set out earlier. We shall also discuss in the light of our findings the previous work carried out with these insects.

2. EXPERIMENTAL METHODS

Examination of the hair pile was carried out by means of freezing microtome sections and whole mounts. Only the hair piles of *Stenelmis crenata* and *Phytobius velatus* were difficult to resolve optically by ordinary methods, and it was not thought worth while to employ specialized techniques as the dimensions of the hair piles were so similar to those of *Aphelocheirus* which had been fully investigated. In view, however, of the importance from the aspect of water protection of the size and spacing of the hairs, accurate estimates were made of:

(i) The distance between adjacent hairs in optical sections of the hair pile.

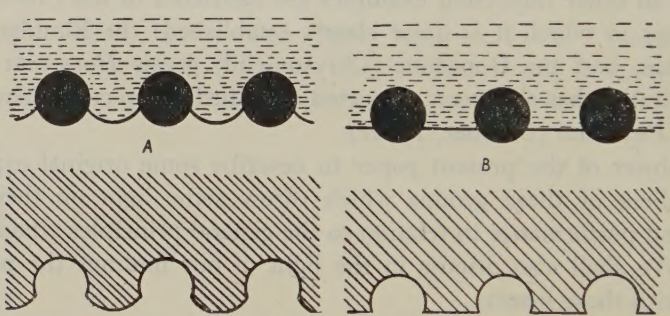
(ii) The number of hairs per unit area. The bases of the hairs were frequently very obvious in whole mounts and could be counted with the aid of a camera lucida.

(iii) The shape, dimensions, and degree of overlap of the hairs in their natural position.

Attempts were made to view the hairs by reflected light, but owing to the diffraction patterns set up by the regularly spaced hairs no reliable information could be obtained from this method. To overcome this a method, described in full elsewhere (Thorpe & Crisp, 1949), was developed to obtain transparent replicas of the plastron air-water interface with the hairs in position; these replicas which were made from a strong solution of gelatin in glycerol revealed the position of each hair at the interface as a hollowed-out relief, while the water menisci between, where they are visible, show as smooth undulations.

The shape of the interface when the hairs are wetted by a liquid having a lower contact angle than water, such as glycerol-gelatin, is not quite the same as would be obtained with a higher contact angle and increased pressure (cf. water under natural conditions). But the difference is confined to regions between the hairs (Text-fig. 1) and does not affect their spacing. Hence we have assumed that the hair spacings obtained by the replica method are not materially different from those existing under natural conditions and this assumption is well justified by the good agreement obtained between measurements from replicas and those made by more direct observations (see lines 8 and 9 in Tables 1–3).

Apart from this replica technique the methods employed were essentially those described in our previous papers. (Thorpe & Crisp, 1947*a*, *b*, *c*, and Crisp & Thorpe, 1947.)



Text-fig. 1. Diagram showing difference in meniscus when the plastron is wetted: A, by a liquid of high contact angle at a positive pressure; and, B, when wetted to the same extent by a liquid of lower contact angle at zero pressure. The upper figures show in section the hairs and the meniscus. The lower figures show the replica derived from it when the hairs are withdrawn after solidification of the liquid.

3. THE DRYOPOIDEA

Natural history

The super family Dryopoidea, which is world-wide in distribution, comprises upwards of 600 species of small beetles distributed among sixty or more genera. Of these many of the Dryopidae (e.g. *Helichus*) and almost all the known species of the sub-family Elminae of the Elmidae (Hinton, 1939) are aquatic, the latter seldom or never leaving the water; while many members of the sub-family Larinae are terrestrial insects living in moist places and remaining under water for relatively short periods, perhaps at most, for a few hours at a time. Brocher (1912*b*) seems

Table 1. *Coleoptera, Dryopoidea. Group II*

	Elminae					Dryopinae
(1) Species	*Elmis maugei*	*Riolus cupreus*	*Elsianus aequalis* and *E. thorpei*	*Macrelmis corisors*	*Pseudomacronychus* sp.	*Helichus substriatus*
(2) Habit	Always submerged, bubble replacement activities (probably also *Coxelmis**)	Always submerged, bubble replacement activities	Not certainly known, probably always submerged	Not certainly known, probably always submerged	Not certainly known, probably always submerged	Frequent visits to surface or access to large bubbles probably necessary†
(3) Shape and condition of hairs	Not completely regular, soft, flexible, overlapping	Not completely regular, soft, flexible, overlapping	Not completely regular, soft, flexible, overlapping	Fairly regular and stiff, 135° bend at tips, much overlapping	Fairly regular, straight, much overlapping	Fairly regular and stiff
(4) Number per sq.cm. (n)	$6–10 \times 10^6$	9×10^6	$1{\cdot}5 \times 10^7$	$5–6 \times 10^6$	4×10^6	$4–5 \times 10^6$
(5) Distance between bases of hairs (l') (cm.)	$3–4 \times 10^{-4}$	3×10^{-4}	$2{\cdot}5–3{\cdot}0 \times 10^{-4}$	$4–4{\cdot}5 \times 10^{-4}$	5×10^{-4}	4×10^{-4}
(6) Angle of inclination of hairs (α) (degrees)	25–35	25–35	30	40	30	60
(7) Overlap of hairs observed in sections, etc.	$\times 2$	$\times 1\frac{1}{2}$	$\times 1\frac{1}{2}$	$\times 2\frac{1}{2}$	$\times 3$	Possibly too stiff to overlap
(8) Calculated distance between hairs at the interface (l) derived from l' and degree of overlap (cm.)	$1{\cdot}5–2 \times 10^{-4}$	2×10^{-4}	$1{\cdot}8–2{\cdot}0 \times 10^{-4}$	$1{\cdot}6–1{\cdot}8 \times 10^{-4}$	$1{\cdot}7 \times 10^{-4}$	4×10^{-4}
(9) Observed distance (l) from replica experiments (cm.)	$1{\cdot}8 \times 10^{-4}$	Thorax, $2{\cdot}0 \times 10^{-4}$ Abdomen, $3{\cdot}5 \times 10^{-4}$	$2{\cdot}3 \times 10^{-4}$	$2{\cdot}0 \times 10^{-4}$	—	—
(10) Radius of hairs (r) (cm.)	$0{\cdot}5 \times 10^{-4}$	$0{\cdot}8 \times 10^{-4}$	$0{\cdot}4 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$	$0{\cdot}8 \times 10^{-4}$
(11) Calculated max. value for Δp (excess pressure in atm.) from $\Delta p = \gamma/r \cos\theta + \sqrt{(\frac{1}{4}l^2 - r^2 \sin^2\theta)}$, with $\theta = 110°$, $\gamma = 72$ (Crisp, 1949)	1·20	Thorax, 1·8 Abdomen, 0·57	0·76	1·06	1·38	0·46
(12) Observed value for excess pressure in atm. (Δp)	*c.* 0·8–1·0	*c.* 0·8–1·0	—	—	—	—
(13) Strength of butyl alcohol to cause full wetting %	6–7	6–7	—	—	—	—
(14) Corresponding contact angle (θ)	*c.* 70°	*c.* 70°	—	—	—	—
(15) Basal respiration rate q (c.c. O_2/sec.)	$1{\cdot}17 \times 10^{-7}$	$4{\cdot}5 \times 10^{-8}$	—	—	—	—
(16) Area of plastron A (cm.^{2})	0·014	0·0126	*aequalis*, 0·062 *thorpei*, 0·028	0·03	0·01	0·06
(17) Assumed value for i_0 (invasion coefficient of oxygen)	$2{\cdot}0 \times 10^{-4}$	$1–2 \times 10^{-4}$	$3{\cdot}0 \times 10^{-4}$	$2{\cdot}4 \times 10^{-4}$	2×10^{-4}	3×10^{-4}
(18) q/Ai_0	$4{\cdot}2 \times 10^{-2}$	$1{\cdot}8–3{\cdot}6 \times 10^{-2}$	—	—	—	—
(19) Farthest extent of plastron from spiracle x_1 (cm.)	0·1	0·1	*aequalis*, 0·1 *thorpei*, 0·06	0·1	0·05	0·1
(20) Thickness of plastron (h) (cm.)	$5–10 \times 10^{-4}$	$5–10 \times 10^{-4}$	$1{\cdot}2 \times 10^{-3}$	$1{\cdot}6 \times 10^{-3}$	2×10^{-3}	$1{\cdot}3 \times 10^{-3}$
(21) Value of $\sqrt{\frac{i_0 x_1^2}{Dh}}$	< 0·2	< 0·2	*aequalis*, 0·16 *thorpei*, 0·09	0·13	0·05	0·15

* Davis (1942). † Harpster (1941).

Table 2. *Coleoptera, Dryopoidea. Groups I and III*

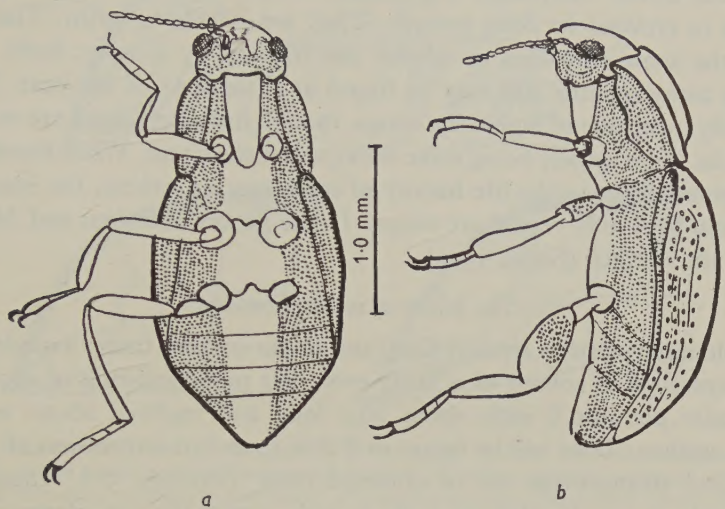
	Group I: Elminae		Group III: Larinae etc.			
(1) Species	*Stenelmis crenata*	*Cylloepus barberi*	*Lara avara*	*Hexanchorus gracilipes*	*Potamodytes* sp.	*Dryops luridus*
(2) Habit	Always submerged, no replacement activities (*S. quadrimoculata*)*	Probably always submerged, but habits not certainly known	Submerged short periods only†	Probably submerged for short periods only‡	Probably submerged for short periods only	Submerged for relatively short periods ‡§
(3) Shape and condition of hairs	Regular, stiff, erect. Bend of 90° at tips	Straight hairs on a complete vestiture of touching or overlapping scales	Fairly regular, curved, stiff. Long and short hairs	Fairly regular, stiff	Fairly regular, stiff	Regular, stiff, two sets long and short
(4) Number per sq.cm. (n)	$2{\cdot}5 \times 10^{8}$	1×10^{8}	3×10^{5}	8×10^{5}	8×10^{5}	Long, 6×10^{4} Short, 6×10^{5}
(5) Distance between bases of hairs (l') (cm.)	6×10^{-5}	1×10^{-4}	$1{\cdot}8 \times 10^{-3}$	$1{\cdot}1 \times 10^{-3}$	$1{\cdot}1 \times 10^{-3}$	Long, 4×10^{-3} Short, $1{\cdot}3 \times 10^{-3}$
(6) Angle of inclination of hairs (α) (degrees)	90	Variable	50	60	60	Long, 45 Short, 30
(7) Overlap of hairs observed in sections, etc.	Negligible	Overlap and touch at various angles	Negligible	Negligible	Negligible	Negligible
(8) Calculated distance between hairs at the interface (l) derived from l' and degree of overlap (cm.)	$5\text{–}6 \times 10^{-5}$	Very approx. 1×10^{-4}	$1{\cdot}8 \times 10^{-3}$	$1{\cdot}1 \times 10^{-3}$	$1{\cdot}1 \times 10^{-3}$	Long, 4×10^{-3} Short, $1{\cdot}3 \times 10^{-3}$
(9) Observed distance (l) from replica experiments (cm.)	—	—	Small, $1{\cdot}3 \times 10^{-3}$ Large, 3×10^{-3}	—	—	—
(10) Radius of hairs (r) (cm.)	1×10^{-5}	$2{\cdot}5 \times 10^{-5}$	1×10^{-4}	1×10^{-4}	1×10^{-4}	Long, 2×10^{-4} Short, 1×10^{-4}
(11) Calculated max. value for Δp (excess pressure in atm.) from $\Delta p = \gamma/r \cos\theta + \sqrt{(\frac{1}{4}l^2 - r^2 \sin^2\theta)}$, with $\theta = 110°$, $\gamma = 72$ (Crisp, 1949)	2·9	2·0	0·08	0·14	0·14	Long, 0·04 Short, 0·12
(12) Observed value for excess pressure in atm. (Δp)	—	—	—	—	—	—
(13) Strength of butyl alcohol to cause full wetting	—	—	—	—	—	—
(14) Corresponding contact angle (θ)	—	—	—	—	—	—
(15) Basal respiration rate q (c.c. O_2/sec.)	—	—	—	—	—	—
(16) Area of plastron A (cm.²)	0·016	0·013	0·13	0·025	0·058	0·05
(17) Assumed value for i_0 as in (17) of Table 1	3×10^{-4}	$1{\cdot}0 \times 10^{-4}$	$4{\cdot}3 \times 10^{-4}$	4×10^{-4}	4×10^{-4}	4×10^{-4}
(18) q/Ai_0	—	—	—	—	—	—
(19) Farthest extent of plastron from spiracle x_1 (cm.)	0·1	0·05	0·15–0·2	0·1	0·15	0·15
(20) Thickness of plastron (h) (cm.)	$3\text{–}5 \times 10^{-4}$	$2{\cdot}5\text{–}5 \times 10^{-4}$	3×10^{-3}	$2{\cdot}5 \times 10^{-3}$	$2{\cdot}5 \times 10^{-3}$	1×10^{-2} and $1{\cdot}7 \times 10^{-2}$
(21) Value of $\sqrt{\frac{i_0 x_1^2}{Dh}}$	0·27	<0·10	<0·16	0·13	0·19	0·23

* Harpster (1944). † Darlington (1929). ‡ Hinton (1936). § Brocher (1913).

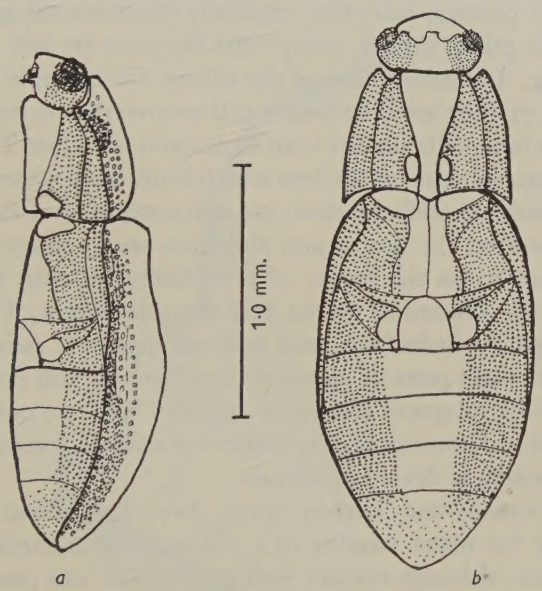
Table 3

Order and family ...	Hemiptera, Naucoridae		Coleoptera, Donaciinae			Coleoptera, Rhynchophora	Coleoptera, Hydrophilidae				
(1) Species	*Aphelocheirus aestivalis*	*A. aestivalis* (sense organ)	*Haemonia mutica*	*H. mutica* (antennae)	*Donacia simplex*	*Phytobius velatus*	*Hydrophilus piceus* (thorax)	*H. piceus* (antennae)	*Hydraena riparia*	*Berosus spinosus*	*Helophorus aquaticus*
(2) Habit	Always submerged	Non-respiratory	Always submerged	—	Terrestrial in neighbourhood of streams	Entirely independent of surface visits	Frequent visits to surface	—	Submerged for considerable periods but has frequent access to surface	Frequent visits to surface	Submerged for considerable periods but has frequent access to surface
(3) Shape and condition of hairs	Regular, stiff, erect, right angle bend at tips	Regular, stiff, slightly curved over, much overlapping	Regular, stiff, 135° bend at tips, overlapping	Regular, stiff, 135° bend at tips, overlapping	Irregular, wavy, flexible	Straight hairs on complete vestiture of overlapping scales	Two sets long, irregular, flexible, bent over parallel to surface and short regular close, set pile	Similar two series of hairs, shorter hairs slightly bent over at tips	Somewhat irregular, stiff, curved at tips	Long and short set of hairs	Long hairs bent horizontal at tips
(4) Number per sq.cm. (n)	$2{\cdot}5 \times 10^{8}$	6×10^{6}	4×10^{6}	$1{\cdot}6 \times 10^{7}$	3×10^{5}	2×10^{8}	Large, 1×10^{4} Small, ?	Large, 2–4×10^{4} Small, 5–15×10^{6}	3–6×10^{6}	Long, 1×10^{6} Short, 1×10^{7}	5×10^{4}
(5) Distance between bases of hairs (l') (cm.)	5–6×10^{-5}	4×10^{-4}	5×10^{-4}	$2{\cdot}5 \times 10^{-4}$	Variable *c.* 2×10^{-3}	7×10^{-5}	Large, 1×10^{-2} Small, ?	Large, $4{\cdot}5$–$7{\cdot}5 \times 10^{-3}$ Small, 4–8×10^{-4}	4–6×10^{-4}	Long, 1×10^{-3} Short, 3×10^{-4}	6×10^{-3}
(6) Angle of inclination of hairs (α) (degrees)	90	30	45	50	20–50	30–50 (on upper surface)	80	Large, 90 Small, 75	60	Long, 60 Short, 45	30
(7) Overlap of hairs observed in sections, etc.	Negligible	Probably 3–4 times when pressed down	×2	×2	Variable	×2–3 (if hairs can be pressed down)	Large, ×2 Small, ?	Little overlap	Probably little	Probably little	—
(8) Calculated distance between hairs at the interface (l) derived from l' and degree of overlap (cm.)	6×10^{-5}	Probably very small	$2{\cdot}5 \times 10^{-4}$	$1{\cdot}25 \times 10^{-4}$	1–2×10^{-3}	2–4×10^{-5}	Large, 5×10^{-3} Small, ?	Large, 4–7×10^{-3} Small, 4–8×10^{-4}	4–6×10^{-4}	Long, 1×10^{-3} Short, 3×10^{-4}	—
(9) Observed distance (l) from replica experiments (cm.)	—	—	$2{\cdot}7 \times 10^{-4}$	—	—	$8{\cdot}5 \times 10^{-5}$	Large, 5×10^{-3} Small, $2{\cdot}3 \times 10^{-3}$	—	—	—	—
(10) Radius of hairs (r) (cm.)	1×10^{-5}	$1{\cdot}25 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$	2×10^{-4}	$1{\cdot}4 \times 10^{-5}$	Large, $3{\cdot}8 \times 10^{-4}$ Small, 2–3×10^{-4}	Large, $7{\cdot}5 \times 10^{-4}$ Small, 1×10^{-4}	$2{\cdot}5 \times 10^{-5}$	Long, $7{\cdot}5 \times 10^{-5}$ Short, $2{\cdot}5 \times 10^{-5}$	2×10^{-4}
(11) Calculated max. value for Δp (excess pressure in atm.) from $\Delta p = \gamma/r \cos\theta + \sqrt{(\frac{1}{4}l^2 - r^2 \sin^2\theta)}$, with $\theta = 110°$, $\gamma = 72$ (Crisp, 1949)	2·9–3·6	(Very high)	$1{\cdot}1$ ($l = 2{\cdot}7 \times 10^{-4}$) $1{\cdot}5$ ($l = 2{\cdot}5 \times 10^{-4}$)	2·0	0·1	2·0 ($l = 8{\cdot}5 \times 10^{-5}$) 7·2 ($l = 4 \times 10^{-5}$)	Large, 0·03 Small, 0·07 0·11 (both set at interface)	Large, 0·03 Small, 0·37	0·3	Long, 0·15 Short, 0·52	—
(12) Observed value for excess pressure in atm. (Δp)	3–4	>5	1·5–2	—	—	>4	—	—	—	—	—
(13) Strength of butyl alcohol to cause full wetting	10–11 %	—	7–9 %	9–10 %	—	10–12 %	—	—	—	—	—
(14) Corresponding contact angle (θ)	*c.* 50°	—	*c.* 55–65°	*c.* 50–55°	—	*c.* 45–55°	—	—	—	—	—
(15) Basal respiration rate q (c.c. O_2/sec.)	$1{\cdot}67 \times 10^{-6}$ (shows asphyxia readily in oxygen want)	—	$1{\cdot}3 \times 10^{-6}$ (can undergo oxygen debt without showing asphyxia)	—	—	$3{\cdot}4 \times 10^{-7}$ (does not show asphyxia readily)	$1{\cdot}5 \times 10^{-4}$ (*Dytiscus marginalis*)	—	—	—	—
(16) Area of plastron A (cm.²)	1·0	—	0·12	—	No true plastron	0·08	3·0	—	0·012	0·095	—
(17) Assumed value for i_0 (invasion coefficient of oxygen)	3×10^{-4}	—	$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	—	$1{\cdot}0 \times 10^{-4}$	3–4×10^{-7}	—	$4{\cdot}3 \times 10^{-4}$	4×10^{-4}	—
(18) q/Ai_0	$0{\cdot}55 \times 10^{-2}$	—	$10{\cdot}8 \times 10^{-2}$	—	—	$4{\cdot}2 \times 10^{-2}$	12–17×10^{-2}	—	—	—	1×10^{-3}
(19) Farthest extent of plastron from spiracle x_1 (cm.)	0·25	—	0·15	0·5	—	0·07	1·2	(Very short antenna)	0·03	0·06	0·15
(20) Thickness of plastron (h) (cm.)	5×10^{-4}	2×10^{-3}	$1{\cdot}4 \times 10^{-3}$	$1{\cdot}0 \times 10^{-3}$	No definite limit	2×10^{-4}	$1{\cdot}6 \times 10^{-2}$ (macroplastron) 5–10×10^{-3} (microplastron)	$1{\cdot}6 \times 10^{-2}$ (macroplastron) 2×10^{-3} (microplastron)	5×10^{-4}	2×10^{-3} (macroplastron) 5×10^{-4} (microplastron)	—
(21) Value of $\sqrt{\frac{i_0 x_1^2}{Dh}}$	0·60	—	0·12	0·47	—	0·16	*c.* 0·75	—	<0·09	<0·17	—

to have been the first to establish the fact that some of these insects possessed functional plastrons.



Text-fig. 2. *Elmis maugei*. *a*, ventral view; *b*, lateral view. The plastron area is indicated by stippling.



Text-fig. 3. *Riolus cupreus*. *a*, lateral view; *b*, ventral view. The plastron area is indicated by stippling.

Elmis maugei and *Riolus cupreus* (Text-figs. 2, 3) are beetles about 2·5 and 2·0 mm. in length respectively. They are inhabitants of more or less swiftly flowing streams and rivers, where they may be found crawling slowly about on submerged rocks, stones, logs or roots or indeed on any surface where the algae which constitute

their main food may be found and which is sufficiently rough to provide a firm grip for their claws. They show a gregarious tendency and often remain clustered motionless in crevices for long periods. They are unable to swim. The larvae are found in the same situations as adults; the life history is long; both larvae and adults live many months and may be found at all seasons of the year. The adults are generally incapable of flight; the wings, though fully developed are weak, flaccid and wettable, there usually being some water under the elytra. While there may be an initial dispersal flight in the life history of some species, others, for example *Elmis quadrinotatus* have only very short wings. In the genera *Stenelmis* and *Macronychus* the wings are vestigial (Segal, 1933).

The plastron in the Elmidae

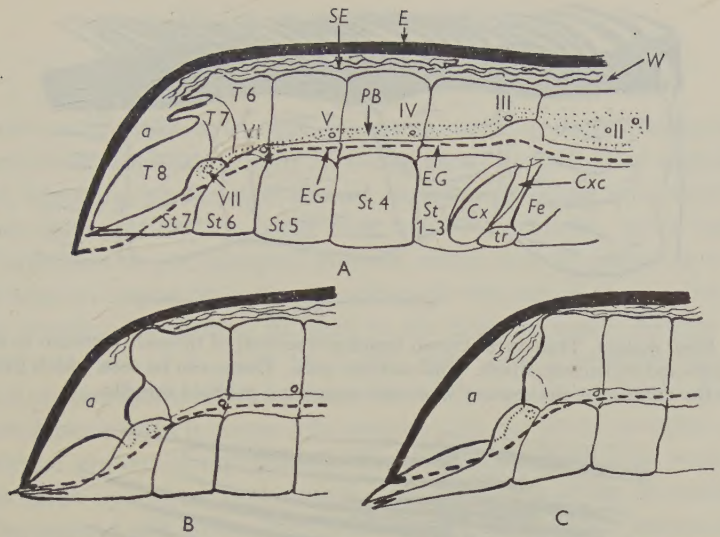
The abdominal plastron areas of *Elmis* and *Riolus* are seen under a moderate power of the microscope to consist of a fairly even hair pile consisting of approximately 100,000 hairs per mm.^{2}, each about 20μ long and inclined about an angle of 40° to the surface. Data will be found in Table 1, and an impression of the general structure and arrangement can be obtained from Text-figs. 5–8. Interspersed at wide intervals among the plastron hairs, as well as over the non-plastron regions of the sterna, are long trichoid sensilla. These may have the same surface properties as the plastron hairs, although as they are isolated from one another and are much larger and stiffer than the plastron hairs they normally penetrate the gas-water interface.

The thoracic hair pile is similar, except that the hairs are less closely spaced and about twice as long. Indeed on parts of the thorax, for example the margins of the epimera (Text-fig. 7), they are even longer still and overlap so much that the lateral spacing of the hairs is actually smaller than on the abdomen (see Table 1, *R. cupreus*). The patches of plastron hairs on the legs which form the brushes used in 'plastron replacement activities', described below, are also composed of the same long hairs.

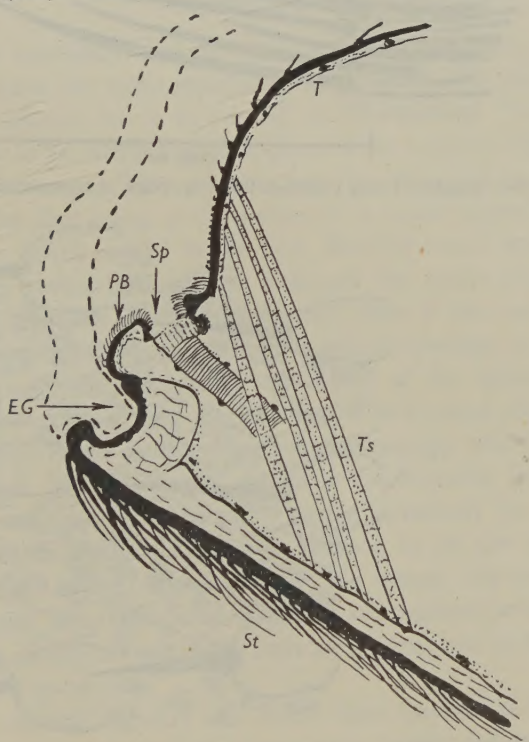
The plastron areas on the thorax and abdomen are in communication with the sub-elytral air space across the groove of articulation between the lateral margins of the elytra and the abdominal sterna and thoracic pleura. This groove is very effectively protected by hydrofuge hairs and constitutes a satisfactory watertight junction. There are eight pairs of spiracles, two thoracic and six abdominal which open into the 'sub-elytral space' (see below, p. 228). The two posterior spiracles are larger than the rest and are supplied by somewhat swollen tracheae, but apart from this the tracheal system is devoid of air sacs.

Both Broeher (1912*b*) and Hinton (1939) have kept *Elmis maugei* alive and apparently healthy for many months in a well-aerated aquarium without having access to the surface, although contact with gas bubbles was possible. In addition, Brocher carried out an experiment in which access even to gas bubbles was prevented for 50 days, still without harm. We have confirmed this with experiments of shorter duration (21 days), but still amply long enough to establish the principle that plastron respiration is effective.

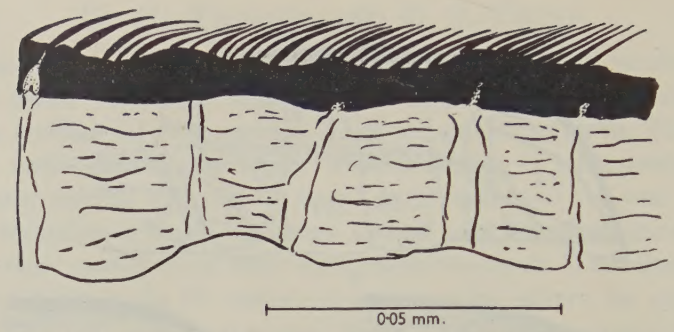
The results of our experiments on the respiration of the Elmidae will be found in Table 4 (p. 232 below). The figures given were obtained from 10 individuals of *E. maugei* and 14 individuals of *R. cupreus*.



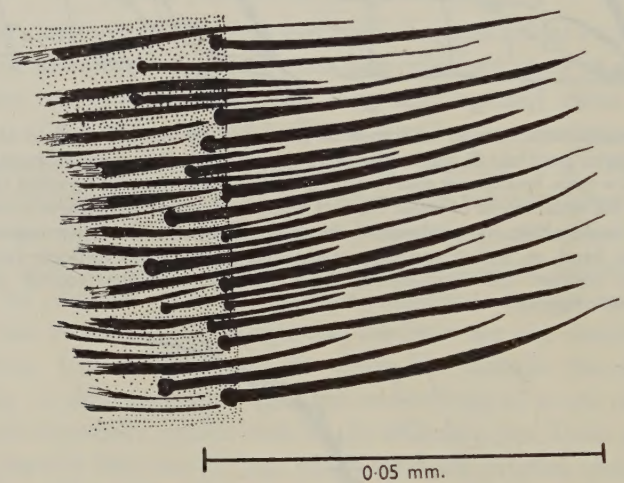
Text-fig. 4. Schematic side view of abdomen of *Elmis maugei* to show method of adjusting specific gravity. A, closed position; B, half expanded position; C, fully expanded position. *a* = air space; *Cx*, *tr* and *Fe* = coxa, trochanter and femur of metathoracic leg; *Cxc* = coxal cavity; *E* = elytron; *EG* = groove for articulation of outer edge of elytron, the position of which outer edge is shown by the heavy broken line; *St* = abdominal sterna as numbered; *T* = abdominal terga as numbered; *PB* = plastron band on which spiracles open; roman numerals indicate spiracles; *W* = wings folded under elytron; *SE* = sub-elytral space containing wings (sternal plastron areas not indicated in this figure).



Text-fig. 5. Transverse section through lateral margin of abdominal segment of *Elmis maugei*. *T* = tergum; *St* = sternum with its plastron hairs; *Sp* = spiracle; *PB* = plastron band; *Ts* = tergo-sternal muscles. Broken line shows position of elytral margin articulating with elytral groove of sternum. *EG* = elytral groove.

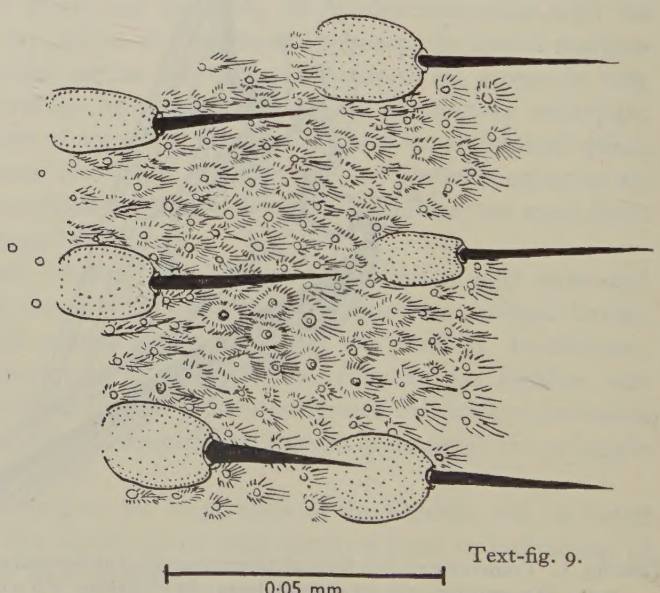


Text-fig. 6. *Elmis maugei*. Transverse frozen unstained section of thoracic sternum to show plastron hairs. Epi- and exocuticle, black. Endocuticle, pale. Ducts can be seen which provide passage through the cuticle for the sensory neurones supplying trichoid sensilla.



Text-fig. 7. *Elmis maugei*. Long plastron hairs on edge of metathoracic epimeron.

Text-fig. 8.



Text-fig. 9.

Text-fig. 8. *Stenelmis crenata*. Metathoracic plastron hairs.
Text-fig. 9. *Cylloepus barberi*. Surface view of plastron area. Note that plastron hairs are borne on a mosaic of scales of varying shapes.

Plastron replacement activities

Elmids are readily kept in aquaria, no special aerating device being necessary as long as there is an adequate supply of living green plants. They normally remain submerged, browsing on the algal film or occasionally biting into plant tissues. If one watches them closely for some time one will probably see a beetle paying particular attention to gas (oxygen) bubbles which may be adhering to the plant, and which tend in course of feeding to adhere to the part of the genal plastron area nearest the mouth. By turning its head to one side the beetle distorts the bubble or pushes it, or part of it, back on to the sides of the prosternum. From this position it is pushed farther back by means of small brushes of plastron hairs situated on the front femora until it comes into contact with the lateral margins of the elytra and the elytral groove of articulation. The bubble may then be sucked into the sub-elytral space by means, presumably, of contractions of the abdominal tergites; or it may be pushed still farther back by means of the plastron patches on the inner surfaces of the femora of the middle and hind legs and in this way distributed generally over the abdominal plastron surface. This type of behaviour has been designated 'plastron replacement activities' by Harpster and others. Its full significance is discussed below in connexion with experimental results.

Hydrostatic control

It is obviously necessary that an insect such as an Elmid, which cannot swim actively, should have the right specific gravity; for if it is either too heavy or too light it will be difficult for it to control its movements. The adjustment of the buoyancy appears to be effected by alteration of the volume of the air space between the elytra and the terga. This process may be regarded as taking place in two distinct stages: the first being in the nature of a coarse adjustment and the second a fine adjustment. With regard to the first, Brocher noted that the wings of *Elmis* and *Stenelmis* are wettable, and that although the space between them and the abdominal terga is mainly occupied by water there is also some air present. It is easy by pinching such insects gently with a pair of forceps, to squeeze out a little air. Bubbles may thus emerge from the region of the metathoracic coxal cavity or from farther down the elytral margins or the abdominal plastron area itself may become distended. Such bubbles are usually quickly absorbed again when the pressure is relaxed. The amount of air varies considerably from one individual to another and is, no doubt, the primary means by which the insect can keep the specific gravity approximately correct in relation to its size and nutritional state. We have found that *Riolus*, apparently by this means, can slowly (2–3 hr.) adjust itself to a change of something under half an atmosphere pressure. Brocher found that *Elmis maugei* usually showed very little air present but we have found specimens in which there is a good deal.

But there is a second, quicker and much finer method of adjustment by which Elmids can increase and control their buoyancy; many species if struggling on the bottom under unfavourable circumstances being able thereby to float passively to

the surface tail first. Thus *Riolus cupreus* if left in a glass dish of water with poor aeration and no vegetation or rough surface to hold on to will soon display a tendency for the abdomen to become lighter than the rest of the body so that the insect 'stands on its head'; soon after this it will float slowly upwards. If on reaching the surface it encounters no foothold it will immediately sink again and this manœuvre may be repeated dozens of times in quick succession. Kaj Berg (1938) has described similar behaviour in *Limnius tuberculatus* which can 'regulate its ascent and descent hydrostatically within a certain limit' and must evidently be 'capable of dilating and contracting the dorsal air supply to a certain degree'.* Observation of *Riolus cupreus* under the binocular microscope, combined with a study of its anatomy, makes clear how this is accomplished (see Text-fig. 4). The abdominal spiracles I–V open into a plastron band (*PB*), which runs down the side of the body just median to the lateral margins of the elytron which articulate very exactly with the lateral margins of the abdominal sterna by means of a deep groove (*EG*). This groove is in functional communication with the plastron band and the ventral plastron area along a considerable part of its length but particularly at the point where the metathoracic coxal cavity impinges upon it and it is the main connecting link between plastron and tracheal systems. It is, of course, via this groove (*EG*) that pressure on the body brings about extrusion of air bubbles. The connexion between groove (*EG*) and plastron band can apparently be closed at will by the action of the abdominal tergosternal muscles (*Ts*, Text-fig. 5), and the region bearing the plastron band is then depressed to form a trough. Each spiracle is equipped with a closing apparatus.† Posteriorly the plastron band is deeper and forms a permanent groove. Into this deeper part open spiracles VI and VII. This deeper region can apparently be sealed off from the rest by distension of the corresponding segment. It opens into an air space (*a*), shown in Text-fig. 4, which is also sealed off from the rest of the subelytral space (*SE*) by means of the same segmental distension.

In the Elmids the whole exoskeleton of the head, thorax, abdominal sterna and elytra (except the last two sterna) forms an extremely strong rigid and well-articulated box. When the head is withdrawn, it fits into the prothorax like a stopper into a bottle, the rigid parts of the exoskeleton forming a watertight vessel whose volume can be changed by the lowering or raising of the abdominal segments 6, 7 and 8 which together form a movable flap hinged at the posterior border of segment 5 and constitute the floor of the air space (*a*) above referred to. Depression of the 8th tergum only will presumably draw more air from the tracheal system (spiracles VI and VII) thus bringing about the preliminary 'up-ending' described above by moving the centre of gravity forward. In *Riolus* the whole flap (i.e. terga and sterna) can be lowered and extended without, at the same time, letting in any water at the apex. This movement not merely shifts the centre of gravity but also has the effect of enlarging the total volume of the system by reducing the air pressure in the space. The rising and falling of the insect in the water can easily be seen to correspond with these movements of segments 6 and 7 (segment 8 is not visible externally).

* We have since confirmed this in *Limnius troglodytes*.

† For a description see Beier (1948).

Elmis maugei displays similar movements of the abdominal flap, but these by themselves are not sufficient to cause the insect to float, they merely assist the preliminary 'up-ending'. In order to float *maugei* has to go one step farther and extrude a bubble of gas which is then retained by the terminal hydrofuge hairs at the tip of the abdomen, while the air space is again expanded. This bubble is attached to the terminal hydrofuge hairs and the insect is then drawn slowly upwards tail first, looking, with its attached bubble, like a little balloon. *E. maugei* cannot always produce a bubble large enough to cause it to float but the phenomenon can be observed in some degree with almost any individual. If the bubble is removed a new one is produced almost immediately—it is often possible to induce the animal to produce a whole series of bubbles in this manner in the space of a few minutes. Brocher supposed this gas to be withdrawn from the spiracles by 'rarefication' of tracheal air but it is clear that the tracheal volume is altogether insufficient for this purpose, for the bubble volume is about 0·03 mm.^{3} and the volume of the main tracheal trunks, it is estimated, cannot be more than 0·003 mm.^{3}. It seems clear that only if the valves of spiracles VI and VII are kept shut and the segments themselves distended, so that the air is not forced back into the tracheal system or the sub-elytral space, is it possible for the insect to extrude its bubble. As soon as one bubble has been squeezed out the insect can then open spiracles VI and VII and by flap movements draw air out from the tracheal system to fill the terminal space once more. This process will, of course, temporarily lower the pressure within the tracheal system, but the air thus withdrawn will rapidly be replaced via the plastron and spiracles I–V from the gases which are in solution in the surrounding water. Thus, provided the plastron is in good working order and provided, of course, that the water contains gas in solution, the process can be repeated indefinitely as bubble after bubble is removed. If, however, the plastron is put out of action by wetting we find that the insects are no longer able to produce a series of bubbles, nor indeed even one bubble.

Gas-retaining properties of the plastron

The Elmids, *Elmis* and *Riolus*, when in normal health may show either a brilliant silver-gilt sheen or a duller golden sheen. If old, or in poor condition, even this golden sheen may be lost in streaks or patches, there being only a faint basic glint where the sheen proper has vanished. Observations under the binocular microscope show that the dull golden sheen is produced by the gas-filled hair pile in exactly the same manner as that of *Aphelocheirus*; when the hair pile becomes wetted a dull glint is left which shows up only in a favourable light and is produced by the hair pile itself without the presence of any gas. The brilliant silver-gilt sheen is, however, without counterpart in *Aphelocheirus*, and is clearly due to the presence of a thicker layer of gas, comparable in its reflecting properties to that of *Hydrophilus*, *Corixa*, etc., where only a very small part of the interface is occupied by the hair tips. Careful observation under vertical illumination indicated that while the tips only of the plastron hairs were present at the interface, the long trichoid sensilla, which are sparsely distributed above the general hair pile, still penetrated the

interface. These conclusions were found later to be in accord with the results of the examination of replica casts of the interface, but owing to the superior wetting qualities of the replica medium the examination of fresh specimens in water by reflected light is more reliable.

We shall distinguish this thicker or enhanced layer of gas from the more tenaciously held dull sheen (plastron) by the term 'macroplastron', although it should be pointed out that this does not imply a separate layer over and above the true plastron, as we first thought, but an expansion of the original (dull) plastron which is held in place by the same plastron hairs, these being inclined at a larger angle to the horizontal.

This macroplastron is unstable and is soon lost if not constantly replaced by the plastron replacement activities referred to above. When a bubble is successfully trapped by *Elmis* on the genal or femoral plastron areas and is pressed against the abdominal plastron, a sudden and striking increase in the brilliance of the sheen may be observed as the bubble is absorbed. This extra brilliance soon disappears when the insects are kept under unfavourable conditions or when they are rendered feeble from any cause.*

The plastron proper, that is to say the gas-filled layer which is held tenaciously and does not require replacement, is in every respect inferior to that of *Aphelocheirus*, as will be seen from a comparison of Tables 1 and 3. In *Elmis* the sheen disappears almost instantly when treated with strong ethyl alcohol, and because of the greater thickness of the gas layer and perhaps of displaced air from the elytral space, bubbles of gas can be seen escaping. With graded strengths of butyl alcohol the sheen resists 5 % but almost disappears in 20 sec. when the strength is increased to 6 % or over; whereas in *Aphelocheirus* a minimum of 8 % is required before any tendency to loss is observed. If we make the assumption that the surface of the hairs is in each case similar, and is hydrocarbon in character, this result suggests that whereas in *Aphelocheirus* the contact angle can be reduced to the order of 60° before wetting occurs, in *Elmis* the hair pile is wetted at a contact angle of 70–75°.

In an earlier publication it was shown that the most efficient structure for a plastron composed of cylindrical hairs was one in which the hairs were arranged as a regular horizontal array (Thorpe & Crisp, 1947*a*). In theory, such a system would not be spontaneously wettable unless the contact angle θ were zero; but in practice any supports, anastomoses, disjunctions or crossings of the hairs, will cause the meniscus to tend to travel into the plastron, and so will cause wetting to occur at some positive value of θ. The greater the number of such defects and the

* M. Beier (1948), in a paper published just as this is going to press, suggests that the 'massage movements', as he calls them, are not plastron replacement activities but aeration activities, the plastron being first distended by increased pressure under the elytra. This distension causes the appearance of the more brilliant silver-gilt sheen and often an actual 'bulging out' of the gaseous plastron which is then kneaded and so ventilated mechanically. This suggestion seems unlikely in view of our experiments, since all Beier's observations seem to accord equally well with our view of the need for constant grooming and fluffing out of the plastron hairs. Moreover, from our present knowledge of the respiratory efficiency of plastrons in general and the Elmid plastron in particular, it seems very improbable that such mechanical aeration would be required whereas the need for plastron grooming and replacement activities seems obvious.

greater the departure from an ideally arranged horizontal array, the higher the value of θ at which spontaneous wetting would occur; until, at the other extreme, a system of straight-sided hairs, vertical or inclined, would wet spontaneously if θ were 90° or less. Since the hairs of *Aphelocheirus* are bent sharply (90°) at the tips, and have therefore only a small region of weakness, while those of *Elmis* are less regular and not sharply curved to offer a horizontal array, thus representing a system of essentially inclined hairs, the greatly inferior water-protecting qualities of *Elmis* can readily be explained. It is possible that *Elmis* and *Riolus* hairs do not offer as high an angle of contact to aqueous liquids as do those of *Aphelocheirus*. On the other hand, as the surface properties are determined by the structure and configuration of the outermost layers of atoms, and since the contact angle to water of 110° for a solid hydrocarbon surface represents probably the highest contact angle attainable in such systems (Adam, 1948) it is very probable that the surface of such highly specialized hairs will in fact offer approximately an angle of 110° in all cases, and that the differences in water-protecting efficiency are solely due to the shape and arrangement of the hair pile. We shall, therefore, assume that the strength of butyl alcohol (or other wetting agent) required to cause spontaneous wetting is an indication of how far the hair pile approaches that of the ideal horizontal array. It is important to note that only the arrangement or geometry of the hair pile and not the scale determines the ease of spontaneous wetting by a wetting agent which reduces the contact angle θ. When wetting occurs under pressure, however, the dimensions of the hair pile become important. It can be seen from the examples in Tables 1 and 3 that there is little correlation between the scale of the hair pile (nos. 8 and 9) and the ease of spontaneous wetting (no. 3).

Although *Elmis*, like *Aphelocheirus*, is a true plastron-respiring insect, it has not the latter insect's margin of safety against wetting under increased pressure. Provided that the gas tension in the environment is maintained, *Elmis* can respire normally, and it can also survive and remain active in oxygen-saturated water (devoid of nitrogen) for at least 40 hr. under conditions where *Notonecta*, an air-store respiring insect, was observed to become waterlogged and moribund in 45 min. (Ege, 1918). In such oxygen-saturated water, however, not only is the macroplastron completely lost, as would be anticipated, but the plastron itself appears a little streaky and ill-defined at the edges.

In gas-free water the plastron is wholly lost in about 2 hr. and does not escape damage for more than about 40 min. after immersion; if immersed in gas-free water under pressure the plastron is lost almost immediately. Clearly, therefore, the plastron is only able to withstand a pressure difference of slightly less than one atmosphere. *Riolus* behaves in a similar fashion.

Another feature of these and many other experiments was the general inability of the insects, when returned to well-aerated water, to recover from the removal of their microplastron, though under some conditions limited recovery can be observed. Wetting by butyl alcohol and cetyl pyridinium bromide seems invariably fatal even though the treatment has been a very short one and is followed by very thorough rinsing. Similarly, none of the insects wetted by a hydrostatic pressure of two atmo-

spheres in gas-free water, survived the treatment. Wetting by gas-free water alone, however, provided it is watched carefully and stopped before the lateral elytral grooves have been involved, does not cause any immediately fatal results and many insects so treated may live for considerable periods and show varying degrees of recovery. To study this matter further, five lots of twelve insects were exposed to gas-free water for 2 hr. and were then placed in a small jar fitted with a bubbler and containing a suitable stone. In some of the jars the stone was left projecting above the surface and in others it was submerged, but in all cases, whether access to the surface was provided or not, there was, of course, an ample supply of air bubbles available to the insects.

Table 4. *Respiration of the Elmidae*

	Elmis maugei	*Riolus cupreus*
Live weight (g.)	0·001	0·00047
Basal oxygen consumption (mm.^{3}/hr./individual)	0·42	0·16
Mean oxygen consumption (c.c./hr./kg.)	422·0	347·0
Area of plastron (mm.^{2})	1·4	1·26
Oxygen uptake per unit area of plastron (mm.^{3}/hr./mm.^{2} surface):		
(*a*) Basal respiration	0·3	0·13
(*b*) Active movement (estimated)	0·9	0·39

Table 5. *Respiration of* Haemonia mutica

Experiment no. ...	1	2	3	4	5	Average of five experiments
Live weight (g.)	0·0125	0·0135	0·010	0·0165	0·011	0·0127
Oxygen consumption (mm.^{3}/hr./individual)	3·33	5·24	4·13	7·25	4·22	4·83
Mean oxygen consumption (c.c./hr./kg. live weight)	280	388	413	440	383	381
Area of plastron including antennae	12 mm.^{2}					
Oxygen uptake (mm.^{3}/hr./mm.^{2} plastron) during rest	0·28	0·437	0·34	0·60	0·35	0·40
Oxygen uptake (mm.^{3}/hr./mm.^{2} plastron) when active (estimated)	—	—	—	—	—	1·20

It was found from these experiments that there is great individual variation but that complete recovery is unusual and that recovery when it occurs does not seem to depend on the opportunity to crawl above the water surface. Indeed it is remarkable how few of these experimental insects showed any propensity to climb up the stone above the water surface; although instead of hiding in the crevices of the stone or clinging with their bodies closely adpressed to the surface as is their normal habit, they would stand 'on tip toes' on the stone in the full water current created by the bubbles, a typical 'asphyxia attitude' which, of course, allows much better aeration of the body surface. Another feature of many of these experimental insects is the persistence of apparently ineffective 'plastron replacement activities'.

Many experiments were performed in which insects which had been wetted by

various means were thoroughly air dried. Whether dead or alive, as they become dry, the plastron areas lose their dark sodden aspect and resume the appearance normal to them when in air. But this restoration to normality is apparent only; as soon as the insect is reimmersed in water the apparently dry plastron at once becomes waterlogged again. In striking contrast to this is the result of wetting with ethyl alcohol, ether, benzene, or xylol. These fluids all spread among the plastron hairs almost instantaneously and, of course, immediately kill the insect. But when they are allowed to evaporate and the animal immersed in water the microplastron again shows its normal golden sheen. Moreover, insects that have once been waterlogged can have the condition of their microplastrons restored to a considerable extent, if not completely, by immersion in ethyl alcohol and subsequent drying. At one stage in the work it was thought that slow asphyxiation might affect the sheen, more than do more violent poisons and strongly surface-active fluids, owing to the possible secretion of surface-active substances by the insect, but this was found later not to be so. Thus, insects with good microplastrons killed with dry gaseous nitrogen show as good a sheen after death as they did in life.

More than one previous observer has suggested that the hydrofuge properties of Elmid plastron hairs are due, not to the nature of the cuticular substance itself but to a glandular secretion which is repeatedly spread over the plastron surface by the grooming motions and 'plastron replacement activities'. While we cannot say that this might not be the case in some Elmidae there seems no need to assume such a method in *Elmis maugei*. Indeed the experiments described above make it seem exceedingly unlikely. The fact that the hydrofuge qualities of the plastron are reduced by contact with water but restored by alcohol, etc., suggests at once that wetting produces a reorientation of the surface molecules tending to reduce the contact angle with water and that the organic solvents have the opposite effect. Certain plant leaves have also been shown to exhibit analogous properties (Fogg, 1947).

This explanation, moreover, is in line with our knowledge of *Aphelocheirus*, *Haemonia* and other plastron insects. In these the plastron is in its most hydrofuge condition on emergence from the last nymphal or the pupal skin as the case may be, and with the vicissitudes of life it becomes progressively less efficient. Indeed the age of individuals of either of these species can be gauged fairly accurately by the state of their plastrons. Elmids are very long-lived, and it is evident that here, too, the same conditions apply and this, no doubt, accounts very largely for the great individual differences in the ability to recover the plastron after wetting.

There remains a curious observation of Brocher's which should be mentioned. Brocher states that if the terminal third, or thereabouts, of the elytron of one side is removed, the beetle loses its sheen on the corresponding sector of the abdomen on the same side only, and he interprets this as being due to the operation upsetting locally the circulation of air under the cover formed by the plastron hairs, with the result that the hairs collapse flat on to the cuticle! We have attempted to repeat this experiment but without success, since we have not been able, by this amputation, to bring about the purely local loss of plastron which Brocher describes.

Status of the plastron in the Elmidae

It will be useful at this stage to compare the typical plastron of the Elmidae, as exhibited by *Elmis* and *Riolus*, with that of *Aphelocheirus*, not merely from the point of view of water resistance, but also from the standpoint of its behaviour. Unlike *Aphelocheirus* the hairs comprising the plastron of the Elmids are flexible and their arrangement, perhaps on this account, appears to be less regular. It is this flexibility which allows the retention of a thicker layer of gas, the macroplastron; for when this increased quantity of gas is present the hairs stand more erect in their unstrained position. Evidently, however, only a small pressure is required to depress the hairs, for under unfavourable conditions of saturation this gas layer is lost. When this takes place there are two important consequences:

(*a*) There is an increasing tendency for the hairs as they are further displaced to return to a more erect position owing to their bending moment. Consequently, a pressure difference Δp is produced equal to that of the surrounding water minus that of the plastron, and this increases with the diminishing volume of the plastron. This pressure difference is exactly balanced by the vertical bending moment of the hairs in their displaced positions.

(*b*) The degree of overlap of the hairs increases, bringing a greater concentration to the interface. As they become more tightly packed in this way the water resistance of the system increases (Thorpe & Crisp, 1947*a*). The loss of the silvery brilliance of the plastron under these conditions may be ascribed to the decrease in free-water surface which provides the internally reflecting surface. Associated with this loss is some loss in respiratory efficiency.

We have shown in a previous publication (Crisp & Thorpe, 1948) that the essential difference between a plastron and an air store lies in the production of such a pressure difference Δp between it and its surroundings, sufficiently high to allow respiration and the maintenance of the plastron space at constant volume. We thus distinguished two possible systems:

(1) in which $\Delta p = 0$ and the volume is variable (air store);

(2) in which Δp is positive and the volume is fixed (plastron).

It will be apparent from the above description that in these Elmids an intermediate type of behaviour is found. The macroplastron behaves essentially as an air-store type of mechanism, being dependent on replacement; but as it diminishes in thickness a pressure difference Δp is set up. When this is great enough to allow equilibration with the surroundings, the volume will become fixed at a value dependent on conditions of saturation, and typical plastron respiration then obtains. In the last analysis, of course, all plastrons have some degree of elasticity and their volume will vary slightly with changes in Δp; the interesting point about *Elmis* is that this variation in volume takes place over a wide range and is a significant feature of the whole process. Indeed it is put to some advantage, for the macroplastron with its greater free-water surface is undoubtedly a more efficient respiratory organ.

Dr M. G. M. Pryor has pointed out to us that a ball of kapok is not readily

penetrated by water on submersion, owing to the hairs bending into the interface and concentrating there into a felty layer. This is a very close analogy to the behaviour of the plastron hairs in Elmids when the macroplastron is being reduced.

The flexible character of the hairs will, however, have another and less desirable consequence. Whenever a system of equidistant, horizontally placed hairs lies at an interface and is subjected to a pressure difference, there is an element of instability, such that if the hairs become displaced from their regular arrangement this displacement will tend to become greater owing to the inequality of pressures on the two sides of the displaced hairs (Crisp, 1949). If this displacement force is less than that of the elastic recovery the hair pile as a whole will be stable—as is almost certainly the case in, say, the stiff hairs of *Aphelocheirus* or *Haemonia* (see below, p. 240)—but if the former is about equal to, or greater than, the latter, as is likely with flexible hairs, the hairs will tend to mat together in clumps and leave bare wetted patches. Such an appearance is very frequently to be seen in the Elmids and is the usual precursor of general invasion of the plastron by water, as would be expected from the analysis given elsewhere (Crisp, 1949).

Thus, although flexible hairs have the advantage of being able to concentrate at the interface and render permeation of water more difficult than would be possible if their erect position were maintained, this advantage is offset to some degree by the tendency of the hairs to clump together, leaving large spaces which are readily wetted.

It is against the background of these limitations that we can see the true significance of the 'plastron replacement activities'. The plastron in such Elmids has normally only a small margin of safety against wetting and, moreover, as the macroplastron space is reduced, the hairs pack into the interface and reduce its efficiency as a respiratory organ; hence the need for the macroplastron to be actively maintained by smearing bubbles of gas over its surface until they are absorbed. Only when this first line of defence breaks down is the insect seriously exposed to the danger of becoming waterlogged. This danger is further reduced by the persistent grooming activities which keep the hairs regularly spaced, prevent clumping, and probably assist in distributing captured bubbles over the plastron surface, thus displacing any small patches of water which may have penetrated. In their ability, when totally submerged, to effect at least partial recovery of the plastron (under well-aerated conditions) the Elmids stand apart from other plastron insects, and this is probably due to their grooming behaviour.

It has been suggested that the grooming activities are a mechanism of spreading some waterproofing secretion over the hairs. We have already given some evidence against the existence of a specific and separate waterproofing wax, and while we cannot prove absolutely that such does not exist, the importance to the prevention of ingress of water of keeping the hairs tidily arranged appears to be a sufficient reason for the existence of this behavioural adaptation.

Elmis and *Riolus* must of course live in well-aerated, preferably moving water; they are not able to go very deep; and they must avoid the risk of remaining long in an environment where there are no gas bubbles available.

Plastron adaptation within the Dryopoidea

Other members of the Dryopoidea show plastron adaptation to a greater or lesser degree, and such detailed observations as we have been able to make and to collect are given in Tables 1 and 2. It should be borne in mind in reading these tables that the calculated maximum pressure to wet the hairs is based on an equation for an idealized array, and is only likely to give values correct in order of magnitude. This is found to be true in those animals which have been subject to experimental investigation; the Elmids *Elmis* and *Riolus*, for instance, are not as efficient in a practical trial as might be expected, whereas *Haemonia* and *Hydrophilus* (Table 3) are more efficient than expected from theory. Nevertheless, the calculated values give a fair prediction and offer a guide in such cases where no experimental evidence is available.

Perusal of Tables 1 and 2 shows that the Dryopoidea studied can be separated into three fairly distinct groups on the basis of hair-pile dimensions and water-protecting efficiency as given by the Δp values.

Group I contains two genera *Stenelmis* and *Cylloepus*, both having extremely numerous plastron hairs of almost ultra-microscopic dimensions. Thus *Stenelmis crenata* with a density of $2{\cdot}5 \times 10^8$ is almost as perfectly adapted a plastron insect as *Aphelocheirus*. *Cylloepus barberi* is interesting in that its plastron hairs are borne upon a complete vestiture of touching or overlapping scales in exactly the same manner as the plastron of the weevil, *Phytobius velatus* (see below, p. 247). It is an American species and practically nothing appears to be on record about its life history. The life history of *Stenelmis crenata* is similarly unknown, but Harpster (1944) has published a paper on *S. quadrimaculata* Horn, and Brocher (1912*b*) gives a few facts about *S. canaliculatus*. It is clear from both authors that members of this genus have a thin gas film over the whole of the ventral and part of the dorsal surface and that they remain permanently submerged. There are apparently no plastron replacement activities. Brocher figures an extremely fine hair pile but from his description it seems doubtful what the nature of the surface is and whether there is actually a hair pile there or not. This is understandable in view of the extremely small size of the hairs which are almost certain to be overlooked unless sections are examined under the highest powers of the microscope. Harpster also fails to detect any hairs in *S. quadrimaculata* and *S. douglasensis*. She describes a finely granular material (probably concretionary) obtained from the ventral surface by scraping and adds 'To this surface the gas layer adheres so closely it cannot be removed by brushing.' Brocher describes the gradual absorption of small gas bubbles adhering to the plastron surface which is presumably brought about by the active decrease of pressure in the sub-elytral space. Harpster assumes that for continued existence beneath the surface oxygen bubbles must be available, but she brings no definite proof of this and if the plastron is, in fact, as it appears to be, as efficient as that of *Aphelocheirus*, oxygen bubbles should be superfluous. Harpster also provides evidence to show that the sub-elytral chamber is not a necessary part of the respiratory mechanism—an observation which it is difficult to reconcile with her other conclusions just quoted. Harpster concludes also that certain solvents

remove the waterproofing substances from the plastron surface, but she does not seem to have allowed for possible reorientation of the surface molecules. Her work on 'substitute plastrons' is based on a misapprehension of the diffusion conditions existing in the plastron; from our approximate calculations on *Aphelocheirus* it is clear that the 'substitute plastron' could not in fact exist in *Stenelmis* for more than a few seconds.

The second group contains insects having plastron hair pile of density between 3×10^6 and $1{\cdot}5 \times 10^7$ per cm.^{2} and includes all the remaining Elmids described, except *Stenelmis crenata* and *Cylloepus barberi*.

Members of the third group containing all the *Dryopinae* and *Larinae*, except *Helichus substriatus*, have a relatively coarse hair pile of density between 6×10^4 and 8×10^5 per cm.^{2}. *Dryops luridus* and *Lara avara* are the only members of this group about which any exact biological information is available.

This series of Dryopoid beetles, ranging from *Dryops* at one extreme to *Stenelmis* at the other, thus provides a very obvious indication of the way in which the fully adapted plastron-bearing insect can have evolved from the riparian form with a hair pile the sole function of which was to enable the insect to enter the water for oviposition or to safeguard it against accidental immersion. There is thus no difficulty in envisaging the series of gradual steps by which the hair pile of a riparian insect could be transformed into the minute plastron structure of *Stenelmis*. Probably much the most difficult and complicated step in this transition process would be the perfect articulation of the sclerites to form the rigid and incompressible box which is so characteristic of the Elminae and which is an essential part of their adaptive organization.

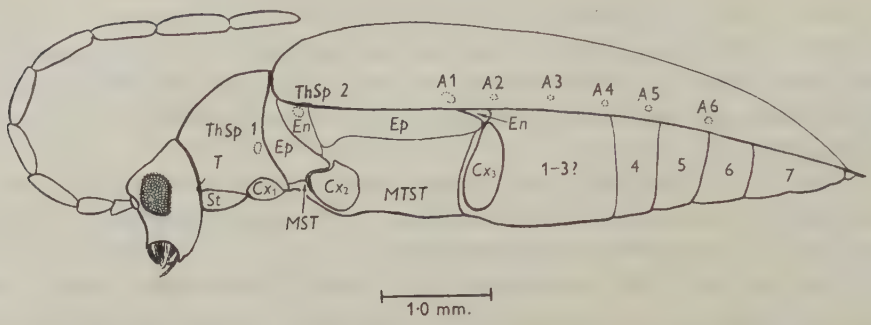
4. *HAEMONIA* (*MACROPLEA*)

Natural history

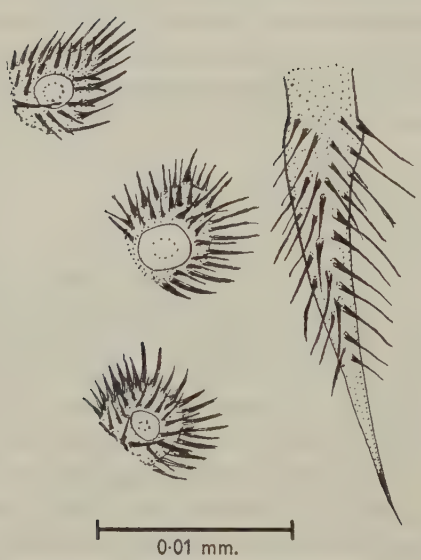
The Chrysomelid beetles of the subfamily Donaciinae are widely known among entomologists and fresh-water biologists, generally because of the remarkable adaptations, first elucidated by von Siebold in 1859 (see Deibel, 1911), whereby their larvae obtain their oxygen from the intercellular gas-containing spaces in the stems and roots of water plants. But whereas *Donacia* and *Haemonia* adults are equally well organized for an existence in air the latter are always found clinging tightly to the submerged stems and foliage of water plants, or walking sluggishly among them and never showing any tendency to come to the surface (e.g. Forel, 1904). J. Deibel's work on the biology and physiology of the adult *Haemonia* is seriously in error and it was not until the pioneer work of Brocher (1912*a*) that it became clear that *Haemonia* adults were indeed plastron insects—though he did not fully grasp the nature of the plastron mechanisms, since he thought that the hair tips were agglutinated to form a membrane through which the gas must diffuse.

Plastron in Haemonia

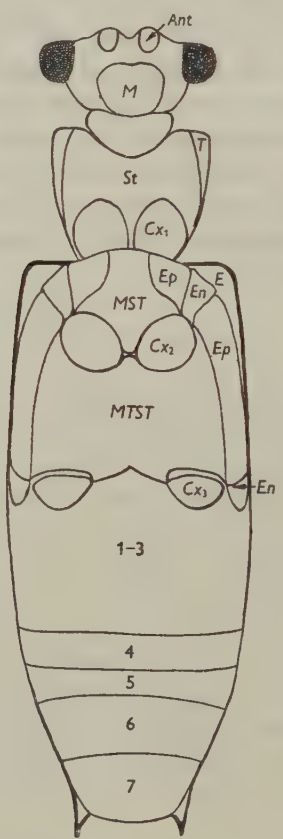
The plastron area of *Haemonia* shows itself as a rich golden sheen covering the whole of the ventral surface of thorax and abdomen, as well as most of the head region below the level of the eyes and also the whole of the long antennae which



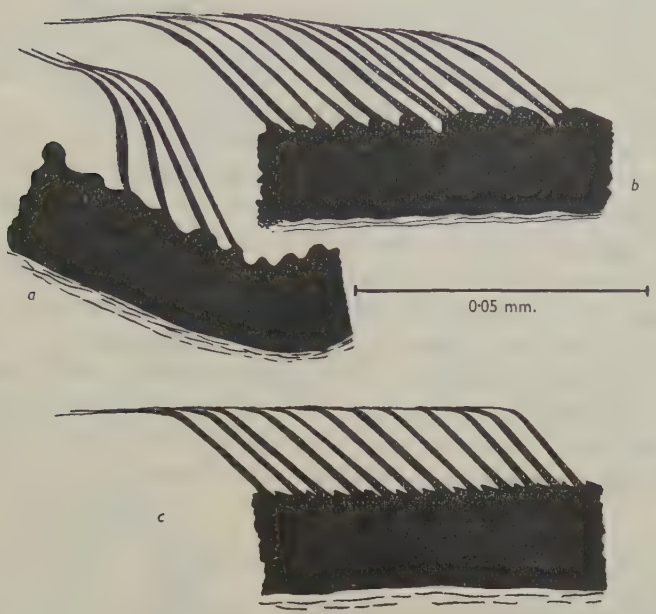
Text-fig. 11. *Haemonia mutica*. Lateral view to show proportions, and positions of the spiracles. *T* = tergum of prothorax; *ThSp* 1 and 2 = 1st and 2nd thoracic spiracles; *St* = prothoracic sternum; Cx_1, Cx_2, Cx_3 = coxae; *Ep* = episternum; *En* = epimeron; *MST* = mesosternum; *MTST* = metasternum; *A* 1–6 = Abdominal spiracles; nos. 1–7 indicate abdominal sterna.



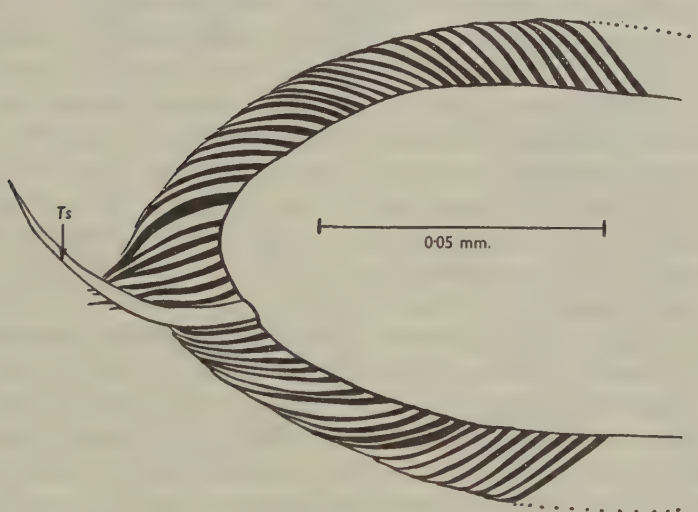
Text-fig. 10. *Cylloepus barberi*. Plastron-bearing scales.



Text-fig. 12. *Haemonia mutica*. Ventral view. Lettering as in Text-fig. 11, with the addition of: *Ant* = antennal socket; *M* = moulds; *E* = edge of elytron.



Text-fig. 13 *a*, *b* and *c*. *Haemonia mutica*. Three characteristic pieces of section through plastron of thoracic sternum. Sections cut with freezing microtome; unstained. Exocuticle only shown; endocuticle and hypodermis omitted.



Text-fig. 14. *Haemonia mutica*. Plastron hairs on tip of last antennal segment. Camera lucida drawing from whole mount in optical section. *Ts* = trichoid sensillum.

are made very conspicuous by their sheen. The sheen does not show the variation in colour and intensity that is so characteristic of *Elmis*, though, as in all plastron insects, it may become worn and patchy with age. The manner of communication between plastron and spiracles is in principle very similar to that described in *Elmis* and can be understood from Text-figs. 11 and 12.

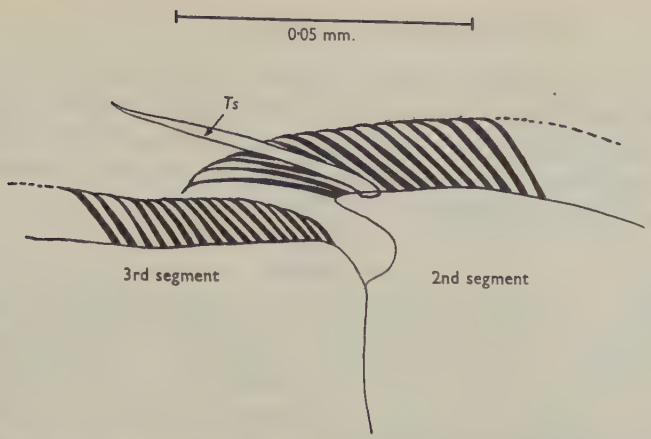
The plastron hair pile of *Haemonia* is remarkably uniform. Its structure and dimensions can be gathered from Text-figs. 14 and 15 and Table 3. It will be seen that while much larger than in *Aphelocheirus* the hairs are the same stiff type having a beautifully adjusted bend of about 130° at the tip. The hairs are rigid and the spacing and adjustment are so perfect that an extremely smooth and even plastron interface results without the extreme degree of overlapping, flexibility and irregularity, and without the resulting tendency to pack and obliterate the air/water surface characteristic of *Elmis* and *Riolus*. Particularly beautiful are the modifications of the hairs at the edges of the plastron surface, notably at the articulation of the antennal joints (see fig. 15) where they are so adjusted as to give sufficient flexibility without allowing a gap so large as to cause risk of wetting.

Resistance to wetting

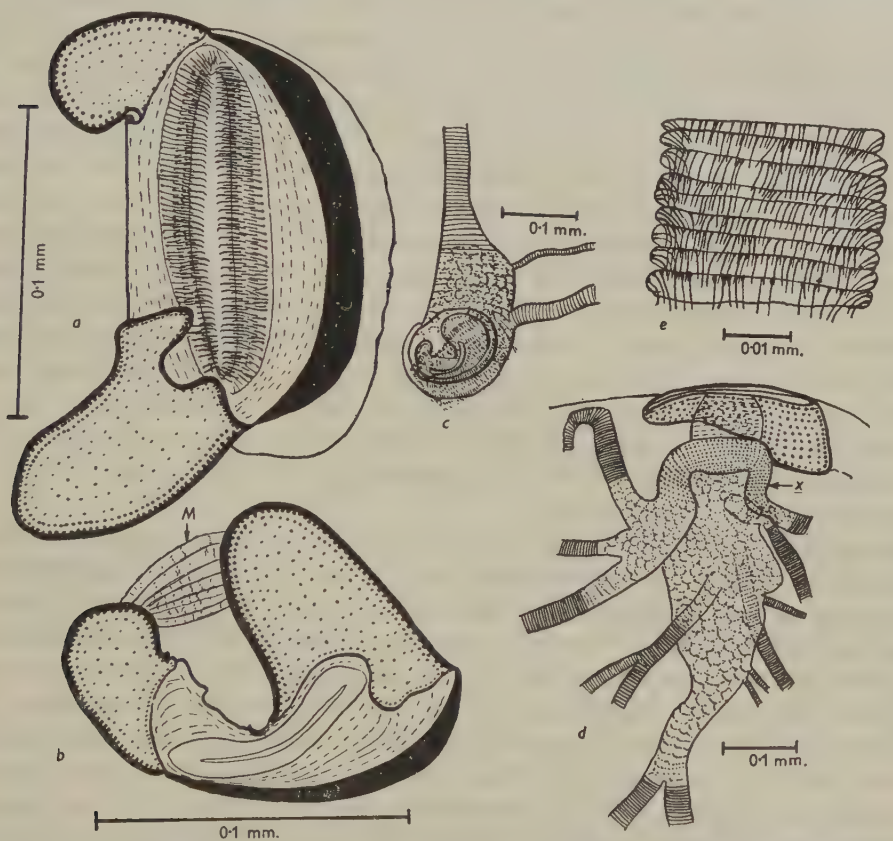
Experiments with butyl alcohol show that the greater part of the hair pile of a typical specimen of *Haemonia mutica*, aged about 2 weeks but not noticeably worn, wets at a concentration of 7–8%. Some small spots may, however, begin to show wetting at a lower value than this, while some areas (e.g. head and parts of thorax) may be much more resistant, even standing up to 9–10 % for a period of several hours.

Wetting of *Haemonia mutica* and *H. appendiculata* commences at from $\frac{1}{2}$ to $\frac{3}{4}$ atm. additional pressure in gas-free water and at 1 atm. proceeds very rapidly. In ordinary air-saturated water about $1\frac{1}{4}$ to 2 atm. additional hydrostatic pressure is required to produce the same effect. As in other plastron insects there is evidence that contact with water may reduce the hydrofuge property of the hair surface. We find that newly emerged adults have a more hydrofuge surface than older insects and very old animals show much plastron deterioration. The exposed side of the plastron hairs tend also to get hydrophile so that an older insect has less difficulty in submerging than a young one; but whether young or old the elytral surface and other non-plastron areas of the body are easily wetted.

It will be seen from Table 3 that the plastron of *Haemonia* is remarkably efficient as a water-protecting mechanism, bearing in mind the fairly large scale of the component hairs. The observed pressure required to break down the resistance is actually greater than that calculated from the observed distance separating the hairs. It is unlikely, in view of the stiffness of the hair bases, that any considerable bending of the hairs takes place under pressure, and the high efficiency must be ascribed to the evenness and regular spacing, rather than to any packing of the hairs as the pressure on them is increased, in marked contrast to the Elmidae (see above, p. 235). For the same reason there is in *Haemonia* neither macroplastron, buoyancy control, nor plastron replacement activities. The plastron is sufficiently resistant to wetting



Text-fig. 15. *Haemonia mutica*. Plastron hairs at junction of 2nd and 3rd antennal segments to show 'articulation' of plastron so as to maintain continuity of plastron surface without too great loss of antennal mobility. Camera lucida drawing from whole mount in optical longitudinal section. *Ts* = trichoid sensillum.



Text-fig. 16. *Haemonia mutica*. Thoracic and abdominal spiracles. *a*, abdominal spiracle open; surface view of whole mount to show closing apparatus and fine hair pile in mouth (hair pile extends over a much larger surface but omitted elsewhere for sake of clarity). Closing muscle omitted. *b*, another abdominal spiracle in closed position. *M* = closing muscle. *c*, abdominal spiracle and associated tracheal enlargement. Area of protective hair pile shown in this figure. *d*, second thoracic spiracle and associated tracheae. *x* = region shown enlarged in fig. *e*. *e*, protective hair pile in tracheal vestibule of 2nd thoracic spiracle.

to allow the insect to withstand any depth of water that it may meet with a reasonable margin of safety, and while the amount of free-water surface available for respiration is not as great as in *Aphelocheirus* (q/A is high in *Haemonia*, Table 3), it is probably adequate for such a sluggish non-swimming insect which is moreover able to undergo oxygen debt without harm.

Respiration

The results of our experiments upon the oxygen requirements of *Haemonia* will be clear from Table 5. It will be seen that variations are not great considering the differences in behaviour. In one set of readings where periods of quiescence alternated with periods of particularly vigorous struggling the rates of oxygen consumption were found to be 2·2 and 5·9 mm.^{3} per hr. respectively. It is probable that an increase by a factor of 3 for extreme activity in such sluggish non-swimming insects as *Haemonia* should be allowed. With actively swimming insects, however, a factor of not less than 10 is required when estimating extreme demands which may be made upon the plastron per unit area (Thorpe & Crisp, 1947*b*). Tables 1–3 give a comparison of plastron insects from this point of view, the significance of which will be considered in the general discussion at the end of the present paper.

Haemonia shows little immediate response to conditions of oxygen scarcity, though it tends to climb upwards when the aquarium is not well aerated, and may occasionally be seen with its antennae floating on the surface of the water, the rest of the animal remaining submerged. If conditions in the aquarium are allowed to get very bad it may even climb out. But it must obviously be able to stand periods of some hours of oxygen deficiency, as clearly it would frequently have to do, living as it does in tangled masses of weed in still water where there must be (Butcher, Pentelow & Woodley, 1930) conditions of severe oxygen want during warm summer nights. We have in fact confirmed that both *Haemonia* and *Donacia semicuprea* can survive many hours in an oxygen-free environment; an ordeal which is fatal to *Aphelocheirus* and *Elmis*—perhaps not surprisingly since both these latter are inhabitants of fast-flowing waters.

A striking feature of the animal's behaviour is the constant waving of the antennae. Although the insects can survive indefinitely without access to gas bubbles these are sometimes seen adhering to the antennae. Brocher thought that these organs played an important part in the bubble capture and absorption. This seems doubtful since the plastron as a whole is efficient and there exists neither the macroplastron nor buoyancy control which make bubble capture particularly desirable in the Elmidae. However, under exceptional conditions which occur for short periods in early morning when, although plants have already begun to produce oxygen bubbles in the sunlight, the main water mass is still oxygen deficient it is possible that antennae can play a useful though minor part in the rapid absorption of oxygen from such bubbles.

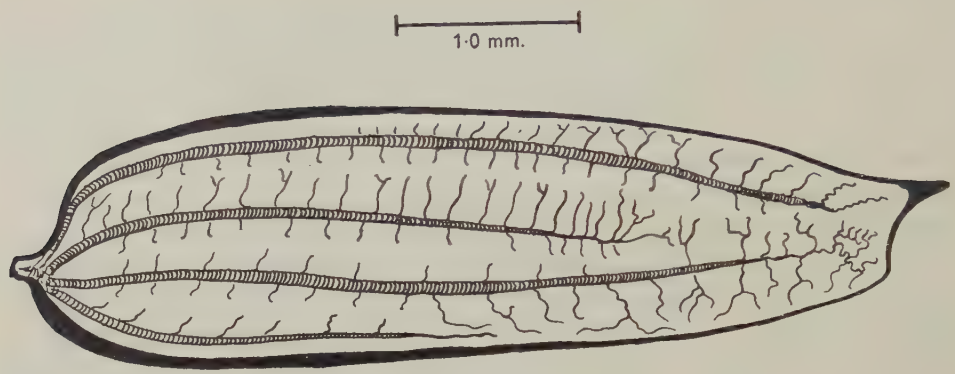
The above observations on antennal movements and the floating of the antennae suggest that these organs may well have a respiratory function, sometimes even serving (Brocher, 1912*b*) to put the insect in direct communication with the

atmosphere. It will be seen from Table 3 that the value of $\sqrt{\frac{i_0 x_1^2}{Dh}}$ when x_1 is the distance to the limit of the antenna, is still small (0·47), hence oxygen uptake is efficient over the whole plastron including the antenna (see below p. 254 and Crisp & Thorpe, 1948). Furthermore, the value of i_0 is dependent on convection and will be much reduced in the region of the antennal plastron when this is in motion, owing to the thinning out of the diffusion boundary layer (Thorpe & Crisp, 1947*a*). Hence oxygen will have a relatively free diffusion path into the antenna, along the gaseous plastron space (where diffusion resistance is very small) and into the spiracles. It will not readily be lost from the abdominal plastron areas even if these are in a region relatively unsaturated in oxygen, because the diffusion shells will there be thicker, the water not being in motion. However, the antennal movements appear not to be dependent on oxygen tension and are probably concerned in mating orientation as well as respiration. Thus, apart from general upward movements under adverse conditions there are no movements that can be regarded as essentially and peculiarly respiratory—no specific movements for plastron aeration, no 'asphyxia attitudes', in fact, no behaviour which gives one any clear and immediate indication of the animal's respiratory state. For this reason, among others, *Haemonia* is a less satisfactory experimental animal than *Elmis* and far less so than *Aphelocheirus*. Yet in its own way it is as perfectly adapted to aquatic respiration as is the latter animal and we have carried out a 'prevention-of-surface-access' experiment in the same apparatus as used for *Aphelocheirus* (Thorpe & Crisp, 1947*a*, p. 240, fig. 7) for 6 months without the animal showing any abnormal plastron deterioration.

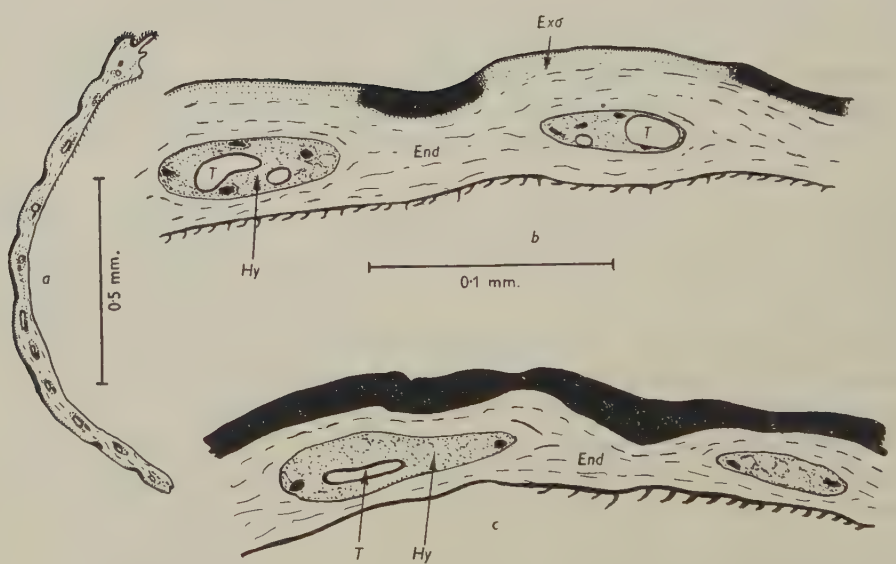
The very small sub-elytral chamber contains air but the elytra are locked together and the wing muscles are degenerate. The wings are complete but with somewhat degenerate venation.

As in *Donacia* there are two pairs of thoracic and seven pairs of abdominal spiracles. Their positions are shown in Text-fig. 11. The first thoracic pair is situated in a plastron-filled groove under the ventro-lateral overhang of the prothorax. The second is similarly protected close to the metathoracic coxae. The remaining spiracles are protected by the elytra. All spiracles are open and with good hydrofuge filter protection and with closing apparatus (Text-fig. 16), except the seventh abdominals which appear closed and rudimentary.

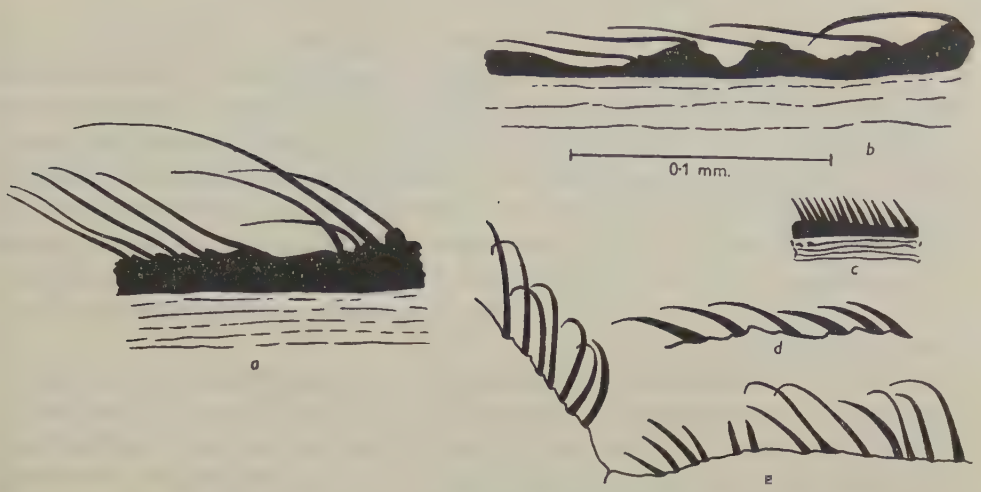
The tracheal system presents no unusual features. There are no true air sacs, though the tracheae just within the spiracles are usually swollen slightly to form a vestibule. The tracheation of the elytra (Text-figs. 17, 18) is often very conspicuous in mounts owing to the relative absence of pigment and the thinness of the cuticle composing the elytral ridges. The whole appearance in sections suggests that the elytral surface may be acting as a kind of tracheal gill, but if this is so comparison with *Donacia* (Text-fig. 18) shows that it is entirely because of the thinness and permeability of the cuticle, and not due to hypertrophy of the tracheal supply.



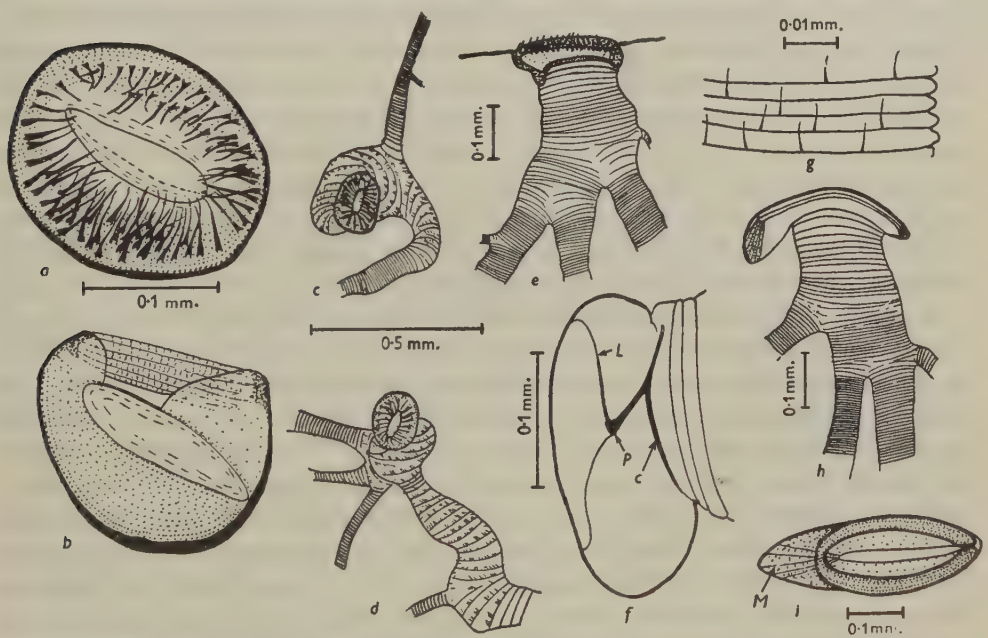
Text-fig. 17. *Haemonia mutica.* Tracheation of elytron.



Text-fig. 18. Transverse sections through elytra of Donaciinae. *a*, transverse section of elytron of *Haemonia mutica* seen under low power. *b*, portion of same under high power. *c*, transverse section of elytron of *Donacia semicuprea* for comparison with *b*. *T* = trachea; *Exo* = exocuticle (where heavily sclerotized exocuticle is indicated in black); *End* = endocuticle; *Hy* = hypodermis.



Text-fig. 19. *Donacia simplex*. Hair pile from various parts of the body for comparison with *Haemonia* plastron hairs. *a* and *b*, transverse section of abdominal sterna. *c*, transverse section of abdominal pleuron. *d*, antenna, last segment. *e*, junction of 1st and 2nd antennal segments.



Text-fig. 20. *Donacia semicuprea*. Thoracic and abdominal spiracles. *a*, abdominal spiracle, surface view of whole mount to show dimensions of protective hairs. *b*, closing apparatus of abdominal spiracle. *c* and *d*, abdominal spiracles with associated tracheal trunks. *e*, 1st thoracic spiracle. *f*, semi-diagrammatic side view of closing apparatus of 1st thoracic spiracle. *c*=closing bar; *L*=lever to which muscle attached; *P*=pivot. *g*, protective hairs on vestibule of 1st thoracic spiracle (compare Text-fig. 16*e*). *h*, 2nd thoracic spiracle and associated tracheae, side view showing closing apparatus. *i*, surface view of 2nd thoracic spiracle. *M*=closing muscle.

Evolution of plastron in Donaciinae

Since the Donaciinae are a very homogeneous group with a highly specialized mode of larval life and respiration it is particularly interesting to consider how the very specialized adaptations concerned with the plastron respiration in the adults may have arisen in the adults of this one genus of the family. *Donacia simplex* and *D. semicuprea* are two common insects of about the same size as, or somewhat larger than, *Haemonia*. They are very liable to the risks of falling into the water and are so adapted that when this happens they can float dry for long periods and, if the conditions of temperature and sunlight are right, can even take flight direct from the water surface.

The difference in structure and arrangement between the hair pile of *Donacia* and *Haemonia* will be clear from Text-figs. 13, 14 and 19 and Table 3. If one tries to submerge a *Donacia* one immediately notices its extreme buoyancy resulting from the air carried as a bubble by the longer of the hydrofuge hairs; when thus immersed the animal is quite helpless. This larger bubble can be brushed off—it would in any case of course soon be lost by the Ege effect—and when it has been removed in this way we are left with a film of air somewhat resembling a poor and streaky plastron to which bubbles still adhere in places. Parts of this plastron can be removed by 6–7 % butyl alcohol; but other parts—isolated streaks and patches, where presumably the long hairs have got matted together—are as resistant to wetting as the plastron of *Haemonia*. There is no reason to suppose that there is any difference in property of the hair surface of the two animals. Since *Donacia* is normally dry the resistance to wetting of its hair must be compared with the newly emerged as yet unwetted *Haemonia*. Once a *Donacia* hair pile has been kept for some hours in contact with water it becomes rather more easily wetted on subsequent occasions. Similarly, because the outer surfaces of the hairs of *Haemonia* are readily wetted there is no need to assume that they were originally chemically or physically different from the rest of the hair; their readiness to wet can be accounted for by the change in surface properties as a result of continuous water contact, the even and regular tips of the hairs preventing the meniscus encroaching farther. Thus there is no need to postulate any mechanism other than the obvious differences of surface density, shape, size and regularity between the hairs of the two animals to account for their difference in behaviour. *Donacia*, in becoming aquatic in the larval stage, has had to adapt itself to survive temporary contact with water in the adult stage. In accomplishing this it has acquired as it were the raw material for the evolution of a plastron mechanism—a hydrofuge hair pile. But it cannot itself be a plastron insect because its hairs are too large, too few, too irregular and incorrectly shaped.

Finally, at least for the Coleoptera, specialization for plastron respiration is probably difficult to combine with the retention of the powers of flight; for a beetle with the substantial sub-elytral air space necessary to house functional wings is likely to be too light for the kind of existence for which plastron respiration is advantageous. The easiest way for it to reduce and control its buoyancy is to reduce the air space by bringing in a certain amount of water. This is what

has happened in both the Elmids and *Haemonia*, and coupled with it the powers of flight have been lost. Although the presence of some water under the elytra would not seem necessarily to result in inability to use the wing, yet if the quantity is at all considerable the opening of the wings must certainly become very much more difficult, causing delay in taking flight. Another result of the partial invasion of this space by water is the necessity of extending the protection afforded by plastron hairs all along the marginal strip or groove and even into the mouths of the spiracles. It is interesting from this point of view to compare these spiracular structures of *Donacia* and *Haemonia* (see Text-figs. 16, 20).

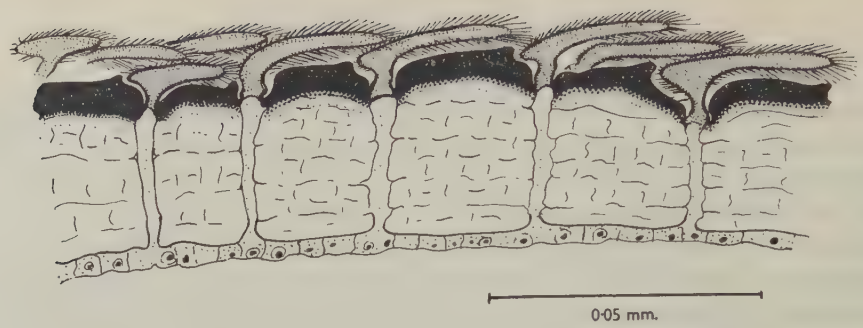
5. AQUATIC RHYNCHOPHORA. *PHYTOBIUS* AND OTHERS

A number of European genera of weevils are known to be aquatic or semi-aquatic. Thus *Tanysphyrus lemnae* (Payk), which is found on duckweed *Lemna* spp., is a lively insect but unable to swim and is helpless when submerged, while *Lixus paraplecticus* will submerge when alarmed, dragging with it a film of air. *Stenopelmus rufinasus* Gyll. specialized for life on the floating fern *Azolla*, is also primarily a surface-dwelling form; but though quite unable to swim it can crawl slowly beneath the water dragging with it a considerable ventral bubble of air. *Phytonomus alismatis* is amphibious though it cannot swim, while within the rare genus *Bagous* (Paulian, 1945) may be found species of every grade of aquatic life. There remains the genus *Phytobius* of which we have had two species for investigation: *P. canaliculatus* Fahr. which is hardly an aquatic insect at all, and *P. velatus* which is an expert swimmer showing aquatic adaptation of the highest order including a virtually 'perfect' plastron mechanism. This species has retained the power of flight.

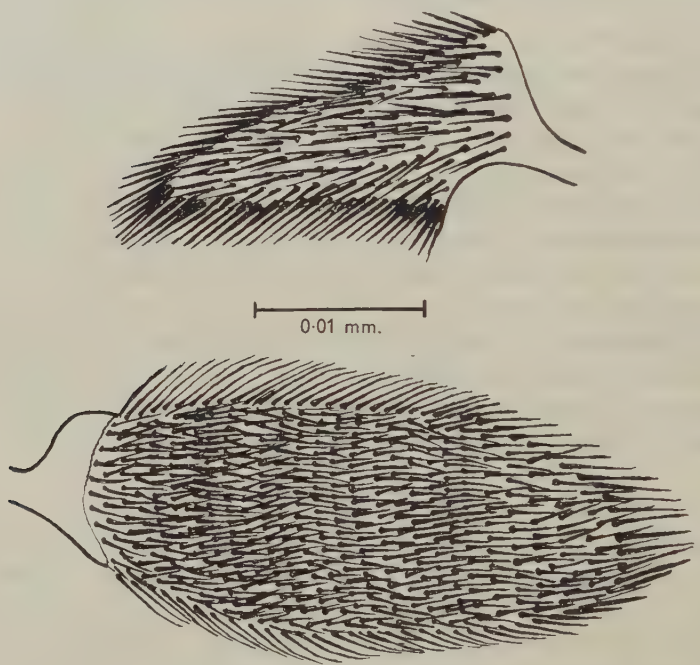
Plastron in Phytobius

Phytobius velatus shows the grey sheen so characteristic of plastron insects over the whole of its ventral surface, the sides of its head and elytra. Examination under a low power shows that *Phytobius*, like so many genera of weevils, bears an armour of flattened scales which is complete over a large part of the body. The scales touch one another almost everywhere and in regions where they are more crowded, actually overlap like roofing tiles. The plastron sheen is seen to be superimposed on these scales. High-power examination shows that each scale is clothed with plastron hairs at a density of $1{\cdot}8–2{\cdot}0 \times 10^8$ per cm.^{2} (Text-figs. 21, 22), and thus a complete plastron is formed. This plastron of course communicates with the abdominal spiracles which open into the sub-elytral space and with the thoracic spiracles which are in the usual sites for the Coleoptera (see section on *Haemonia* above, p. 238) and which are, of course, in this case particularly well protected under the roofing plastron. The tracheal system shows no special features of note. The sub-elytral space is small and contains no water.

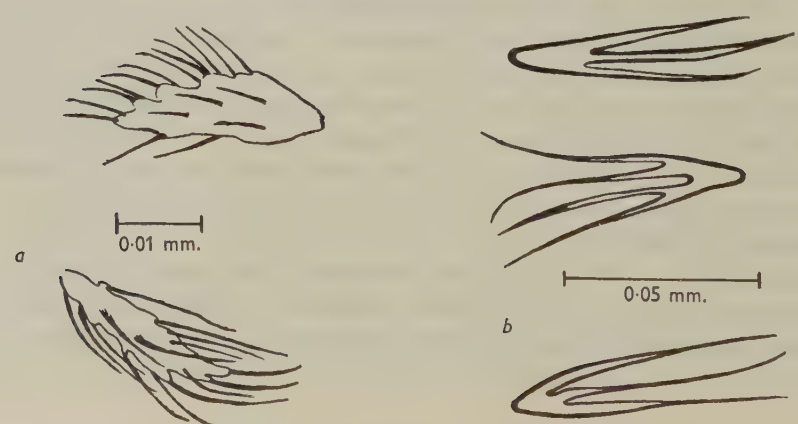
Compared to the other Coleoptera described in this paper the plastron is held very tenaciously. Subjection to 4 atm. pressure in gas-free water produces no noticeable change in the sheen though such pressure kills the insects by dislocation of the neck region.



Text-fig. 21. *Phytobius velatus*. Longitudinal section through abdominal sternum showing scales bearing plastron hairs. Exocuticle black.



Text-fig. 22. *Phytobius velatus*. Plastron-bearing scales from abdomen.



Text-fig. 23. *a, Tanysphyrus lemnae*. Scales from abdomen. *b, Lixus paraplecticus*. Scales from abdomen. A fen species adapted for temporary submergence.

Butyl alcohol solution of 10 % ($\theta = 50–55°$) is required before any extensive displacement of plastron takes place and even at 12 % ($\theta = 40–45°$) there are odd scales and patches of scales which seem yet to retain more or less full sheen. Gas-free water alone causes no effect.

The plastron of *Phytobius* is thus just as efficient as a water-protecting mechanism, and its component hairs are of the same order of magnitude as those of the two other most perfectly adapted plastron insects—*Aphelocheirus* and *Stenelmis crenata*. There is one important difference which is revealed very clearly by replica experiments, namely that under increased pressure or the action of wetting agents the plastron tends to become discontinuous before the plastron-bearing scales are actually wetted. This is because the meniscus tends to invade the larger spaces between the scales more readily than it does between the hairs on any one scale, leaving each scale as a hydrofuge island. The gradual process of wetting can be observed very well in some replica specimens, in the first stage scarcely any unevenness is apparent in the interface, the hair casts being faintly visible where they hold up the water surface at the tip of each scale; with a greater degree of wetting most of the scale leaves an imprint and between the scales the interface is strongly undulated; finally the interface comes right down to the cuticle with the scale still unwetted, sometimes being so completely surrounded that it is plucked out when the replica is removed.

In *Stenopelmus* and *Tanysphyrus* the scales are more widely spaced and are easily isolated when exposed to a mild wetting solution or presumably to pressure (see Plate I, *d*). They are efficient in protecting the insect from occasional immersion in the water from which it can crawl unwetted, but they would not form a reliable plastron. Nevertheless, each hair-covered scale is extremely hard to wet. *Phytobius canaliculatus*, which cannot swim and does not submerge readily, has scales like those of *velatus* but more widely spaced and irregular—thus wetting irregularly and having, when immersed, an inconvenient and irregular bubble to drag about with it. The evolution of a plastron insect like *Phytobius velatus* is easy to visualize. Once such scales are developed, they could, simply by closer approximation to each other, come to give a continuous plastron surface communicating with the sub-elytral space and thence to the spiracles. The fact that most weevils have scales, or similar structures, together with the frequent and sporadic development of cuticular hairs, makes such a course of evolution probable.

Respiration and behaviour

The main facts about oxygen requirements and proportional plastron area are given in Table 3 and are based on the following observations:

Average weight (ten individuals) = 0·00336 g.

Oxygen uptake of resting weevil (average of eight) = 1·243 mm.^{3}/hr. = 372 c.c./kg./hr.

Area of plastron = 0·08 cm.^{2}.

Good steady swimmers though *Phytobius* are, they have no performance comparable to the swift darts of *Aphelocheirus* in the rapid current of its normal environment, but are obviously more active animals than *Haemonia*. If our factors of

10 and 3 respectively for respiration during vigorous activity compared with the basal respiration rate were correct for these latter insects, a factor of 5–7 would seem to be more reasonable for *Phytobius*.

Just as with *Haemonia*, the other still-water plastron insect, so *Phytobius* is capable of remarkable resistance to oxygen lack. Thus, *P. velatus* kept in a flask of gas-free water ceases to be able to swim actively after $1\frac{1}{2}$ hr. but can still walk normally. Even after another $5\frac{1}{2}$ hr. in this oxygen-free environment the insects are still walking fairly actively and swimming is resumed at very nearly the normal rate and degree of co-ordination within 10 min. after return to air-saturated water.

The whole cuticle of the insect is extraordinarily thick and tough (Text-fig. 21) constituting as in Elmids a more or less rigid box. The insect can slowly make very slight changes in its specific gravity, presumably by making a slow change in the volume of the sub-elytral space, but no bubbles are produced externally and the insect when in a pressure jar appears entirely unresponsive to changes in pressure, either positive or negative, of the order of 35 cm. of mercury. The insects take no interest in bubbles and there is nothing in the nature of plastron grooming or plastron replacement activities. A week in a well-aerated aquarium, but where surface access is denied, causes no changes in behaviour or ill-effect of any kind.

6. DISCUSSION

The accompanying tables, 1–3, give all the available data on those plastron-bearing insects or related species which we have been able to examine. It will now be possible on the basis of these data to make a general survey and comparison of them all.

From the standpoint of resistance to water penetration there appear to be three important aspects: (1) arrangement and regularity of the hairs, (2) rigidity, (3) scale of the hair-pile.

(1) We have discussed here and in an earlier publication (Crisp & Thorpe, 1948) how the arrangement of the hairs is of the utmost importance, particularly when the contact angle is less than 90°, and have shown that of all the simple arrangements a regular array of parallel equidistant hairs tangential to the surface probably offers the best resistance to penetration for all contact angles.

This is adhered to remarkably closely by all the insects which we have studied, and indeed the more perfectly adapted plastron insects show this even, regular, unidirectional hair pile to a high degree. Where the hairs arise from the surface at large angle (α) to the horizontal the more specialized plastron insects such as *Aphelocheirus*, *Stenelmis crenata*, and *Haemonia mutica* exhibit a sharp bend in direction at the tip so that the distal part of the hair lies exactly along the plastron surface parallel and coplanar with its neighbours. In these insects the hairs are stiff, and their shape and arrangement are determined during development and not by surface forces which might come into effect when the structure first makes contact with the external medium.

In many of the Elminae (Group II, Table 1) the hairs are relatively long, and in dry specimens have a woolly appearance; while in section they are often apparently

rather irregularly waved. Examination of replicas made from fresh animals show, however, that the arrangement is in the main parallel and tangential. The low angle (α) of the hair bases together with the gentle curvature and more flexible texture makes this alinement possible; and it is assisted in many species by the beautiful behavioural adaptations of grooming described above.

The important exceptions to such a parallel arrangement of the plastron hairs may be divided into three categories: (*a*) Those insects which have a plastron borne on a vestiture of overlapping scales. The limitations of the discontinuous substratum of scales which carries the hairs, the necessity to bridge the relatively larger gaps between the scales, and perhaps the more complex developmental forces operating in the cuticle, may all obscure the simple arrangement of the hairs in a parallel series. In *Phytobius velatus* replicas the hairs are seen to be all arranged parallel to the long axis of the scale and are gently curved to the tip so as to lie along the surface; and since the scales are approximately parallel the plastron arrangement is not far from ideal. In *Cylloepus barberi* the structure is less easy to see, but each scale seems to have a group of stiff hairs radiating from an imaginary point below the centre of the scale in all directions over its upper surface, meeting and criss-crossing with those of neighbouring scales at the periphery. Since only the hairs which bridge these spaces between the scales are vitally important in maintaining the plastron, and these are horizontal (though not parallel), the structure is a fairly efficient one. The minuteness of the hairs probably more than compensates for any defect due to this rather less regular arrangement. (*b*) Those insects in which a set of shorter hairs is borne within a longer and less regular set. The irregular outer hairs form a crude felt-work holding a considerable volume of gas; these are pressed down on to the more regular and shorter hairs when gas store begins to fail. Thus the real water-protecting mechanism is this set of shorter hairs which are usually regular and lie parallel along the interface. An excellent example of this is shown by *Hydrophilus* where the two sets of hairs are quite distinct, and the inner set is very beautifully regular (Plate I, *a*). Other examples of this double set of hairs occur in *Dryops luridus*, *Lara avara* and *Berosus spinosus*. (*c*) Those insects which are not truly aquatic but are protected from accidental submergence (e.g. *Stenopelmus*, *Donacia simplex*). Perhaps in this category should be included simple hairs on a great many insects, where they prevent complete wetting and loss of function. The presence of hairs on the wings of many insects may be particularly important to prevent them from being matted or glued together on to the body.

(2) The rigidity of the hair is important because of the possibility of distortion to give a structure of better or poorer waterproofing quality. When the plastron space is maintained at a pressure lower than that outside, a resultant downward force acts on the plastron hairs which must be mechanically counterbalanced by their bending moment. If the pressure difference is increased and the hairs still remain unwetted, the bending moment must withstand an increased pressure, and the hairs be forced farther down; with short stiff hairs having a large bending moment the deformation is negligible, but with longer flexible hairs the moment is feeble, and bending may be considerable, especially when the angle α is small. The result

is to bring more of the hair into the interface and thus decrease the effective distance between the hairs and increase the water protection afforded. The hairs are nearly always tapered, at least to some extent; this makes the moment small at first, but increasing as the hair becomes more deformed; thus as the hair bends and the waterproofing is improved, the mechanical resistance of the plastron to pressure also increases. Such a mechanism is found in the majority of Elmid beetles and its advantages and disadvantages have been described above, but even in such insects as *Aphelocheirus* or *Haemonia*, where the hairs are stiff, bending may (and in the former species does) occur under abnormal conditions (Thorpe & Crisp, 1947*a*). Normally, however, we have shown that the hairs of such insects remain sensibly in the same position; the large value of the angle of inclination (α) and the stout bases of the hairs clearly offer good resistance to bending.

There exists also a tendency for the hairs to mat together sideways when they act as a water-protecting mechanism and are subjected to pressure (Crisp, 1949), and this may be a disadvantage when the hairs are flexible, since the lateral bending moment may be insufficient to prevent large spaces being formed through which water can readily penetrate. This tendency is somewhat different from the well-known matting which occurs in hair, fabrics, etc., when subjected to incomplete surface wetting without the action of pressure, but resembles the latter phenomenon in requiring a degree of rigidity—though perhaps not so large—in order to withstand unbalanced lateral forces. If this rigidity is insufficient, as perhaps in many Elmids, the insect may actively maintain the regularity of its plastron by grooming.*

(3) Resistance to water penetration increases linearly with the scale of the hair pile, hence other features being equal, the efficiency can be gauged approximately from the density of the hairs. Since all the plastron insects we have studied have a hair pile essentially similar in arrangement and regularity the calculated values for Δp in Tables 1–3 are a reasonable guide in comparing the relative efficiencies. The observed penetration pressures are given where these are known. As shown above, the strength of butyl alcohol required to wet the hairs does not give information as

* We have also found recently that *Hydrophilus* regularly grooms itself, approximately every 7 or 10 days. This operation is not done underwater, as in Elmids, nor was it ever observed when we ourselves kept *Hydrophilus* in a small aquarium with stones projecting, but our attention was drawn to it by Dr M. G. M. Pryor who noticed *Hydrophilus* climbing the stems of growing reeds in a large aquarium, which presumably corresponded more closely to its natural habitat. The beetle hangs upside down by its hind legs several inches above the water surface, combing and grooming itself for an hour or more. The metathoracic plastron is combed by the spurs on the tibiae of the 2nd pair of legs, the mesothoracic with the tibiae of the front legs, the antennae between the tibia and femur of the front legs, and the eye region, side of head, and antennal groove are brushed by the tarsi of the front legs. The region between the eyes is cleaned by the proximal segments of the maxillary palp, and both pairs of palps are waved about during the process. The prothorax and head show great mobility and the front legs are occasionally rubbed together as if to remove debris. Dr Pryor reports that sometimes the abdominal sterna, though lacking plastron, are groomed by the hind legs, the insect clinging by its middle legs. They will on occasion groom in almost any attitude, even when on the back lying in the mud. Perhaps there is a risk in *Hydrophilus* with its very coarse hair pile, that water invasion may occur, and the periodic grooming out of the water will overcome this. There is no evidence of any hydrofuge secretion being applied in the process. It is interesting that Wesenberg-Lund (1943, p. 340) reports his inability to keep this species alive through the winter, all individuals becoming waterlogged and dying in February or March. Perhaps this is due to lack of reed stems or other vegetation in the aquarium suitable for climbing out of the water.

to the efficiency of resisting penetration under pressure, but indicates the degree of contamination and decrease in contact angle tolerated by the plastron; this is not dependent on the scale of the hair pile but rather on its arrangement.

Aquatic and semi-aquatic insects which bear hydrofuge hairs may be conveniently grouped in four series, the first three corresponding to the three groups of the Dryopoid beetles (see pp. 18–19 above).

Those of the first series (see Tables 2 and 3) have a density of 10^8 hairs/cm.^{2} and can probably all withstand a pressure of > 2 atm. without wetting. The plastron is very thin and does not afford any reserve of oxygen but functions simply as a gill. This series comprises Group I of the Dryopoids, *Aphelocheirus* and *Phytobius*, and represent the most perfectly developed plastron-respiring insects.

The second series contains the Dryopoids of Group II and *Haemonia* (see Tables 1 and 3); they have 10^6–10^8 hairs/cm.^{2} and withstand a pressure of 0·5–2 atm. *Haemonia* differs from the Dryopoids in having stiff and extremely regular hairs. The plastron space is thicker than in the previous series, but is not sufficient to encumber the insect on account of its buoyancy, nor is it sufficient to offer more than a small reserve of oxygen even when, as in the Elmids, it is expanded into a 'macroplastron', and actively maintained. As far as is known the insects in both these series have functionally perfect plastrons and, with the probable exception of *Helichus substriatus*, do not require to come to the surface.

In the third series containing *Hydrophilus*, *Hydraena*, *Berosus spinosus*, and the Dryopoid Group III (see Tables 2 and 3), the penetration pressure is less than 0·5 atm. and there are only 10^5–10^6 hairs/cm.^{2}. The plastron in these insects is of considerable volume and acts as much as an air store as a gill, necessitating surface visits and increasing the buoyancy so that the animals have to swim down and cling to the substratum. It is therefore questionable whether they should be strictly regarded as plastron insects, since they do not conform to the strict definition (Crisp & Thorpe, 1948).

The last series contains those insects without a regular plastron at all, where the hairs only offer protection against accidental wetting, and the plastron space if it exists does not communicate functionally with the spiracle. Many insects might be placed in this group with *Donacia* and *Stenopelmus*.

The efficiency of the plastron as a respiratory organ has only been investigated experimentally in the case of *Aphelocheirus aestivalis* (Thorpe & Crisp, 1947*b*), but it is possible to make certain reasonable deductions from our measurements on the number and configuration of the hairs. The concept of invasion coefficient has already been employed as a rough indication of gas exchange conditions at an air-water surface, and this will be employed again using Krogh's value as a basis. It should be borne in mind that if the convection is increased or the dimensions of the structure decreased, the invasion coefficient will be greater, perhaps much greater. Krogh's value is in each case multiplied by a factor $1-2r/l$ (where l is the distance between the hairs and r their radius) to allow for the invasion being restricted to the spaces between the hairs.

When the respiration rate q is known the oxygen-tension drop into the plastron

q/Ai_0 can be calculated. For reasonable efficiency this should be considerably less than 0·2 atm., the oxygen tension of air saturated water. This is seen to be the case for all the permanently submerged insects, though the least active, *Haemonia*, has apparently the smallest margin of safety, while *Aphelocheirus*, which responds rapidly to oxygen want and cannot go for long into debt for oxygen has the widest safety factor.

Hydrophilus, however, seems to have scarcely sufficient plastron area for the needs of so large an insect, but in any event it is not dependent on its plastron but has to make periodic visits to the surface.

A second requirement for respiratory efficiency of the plastron is that the drop in tension along the plastron should be small. We have shown (Crisp & Thorpe, 1948) that where i_0 = the invasion coefficient of oxygen, x = the furthest extent of the plastron from the spiracles, D = the effective diffusion constant of oxygen in the plastron space and h = the thickness of the plastron, the function $\sqrt{\frac{i_0 x_1^2}{Dh}}$ determines the *shape* of the curve of distribution of partial pressures within the plastron; or, in other words, the efficiency of the plastron as a respiratory structure for a given mean drop in partial pressure between the outside medium and the spiracles. If this function is less than 1 the whole plastron is effective as a gill. Thus in all the plastron insects here described this appears to be true.

The limitations imposed by the structure and efficiency of the plastron are well reflected in the habits and natural environment of the insects. All those in series I, with the most highly developed plastron, are insects which remain completely submerged, never so far as we are aware, requiring to come to the surface unless the oxygen in solution is insufficient. They are all heavier than water, living on plants or on the bottom and not possessing any elaborate buoyancy control. *Aphelocheirus* is known to descend to considerable depths.

The second series also comprises completely submerged insects, but the plastron is rather less efficient. Most of the Elmids have an elaborate buoyancy control enabling them to come to the surface when conditions are unsatisfactory. Owing to the rather poorer efficiency of the plastron they cannot descend to any considerable depth in safety, and though in clean water they might descend 10 m. or so, when contamination is present causing a lowering of contact angle this limit would be a good deal less. Indicative of the lower efficiency of the plastron is the existence of behavioural adaptations in this group to assist respiration and in the Elmids gas is actively included in the plastron when possible. In these two series of fully developed plastron-bearing insects, all those from sluggish and stagnant waters are capable of going into considerable debt for oxygen while still remaining active.

The third series contains insects not completely adapted to life below the surface but which must make continuous visits thereto; their buoyancy assists them in this but probably inconveniences their movements below water. There are a number of interesting features in individual members—the specialized aerating mechanism on the antennae of *Hydrophilus*, the ability of *Hydraena* sp.to walk upside down on the surface film of the water, the difficulty experienced by some of them in breaking the surface film (e.g. *Dryops*) so that they must enter the water by crawling down the stems

of plants—and on the whole this series shows a continuous range from almost completely aquatic forms to those which only occasionally enter the water.

In this series are a number which have a double hair pile; the hairs of the outer set are readily pressed down on the inner which give a more complete water protection. The outer set holds a fairly generous air store, which is also of course able to exchange and absorb oxygen from the water as it becomes depleted, but will gradually lose nitrogen by the 'Ege effect'. Eventually the inner set of hairs will be brought into play and, provided the animal is not sufficiently deep to cause wetting of the hairs, the normal type of plastron respiration could go on. It should be noted that the limiting depth corresponding to the penetration pressure is not the limiting depth to which the animal might descend if its outer plastron were full of gas, since the compression of this gas would also assist in preventing the interface from invading past the hair pile. It is, however, the limiting depth for continued submergence when the animal is entirely dependent on plastron respiration. Hence, if the outer hairs contain a volume of gas large in comparison with the tracheal volume, this macroplastron will enable the insect to go to greater depths than would otherwise be possible, and will give it an increased margin of safety. This fact should be borne in mind when considering the rather low values of Δp for this group of insects. The macroplastron here is a definite structure and is not a mere increase in the amount of gas in the plastron as with the Elmids. However, the added safety and better respiratory conditions resulting from the actively maintained macroplastron is completely analogous.

The fourth series comprises only those insects which, owing to their proximity to water, require some protection against wetting so that if they should accidentally enter they can readily crawl out or take flight directly. This requires protection against wetting under zero pressure and is more akin to 'rain-proofing' than protection from penetration.

If we now consider the problem met by insects, which are not adapted for life below the water surface, but which are exposed to the risk of accidental or partial wetting we shall see that the problem is essentially a different one. This difference between resistance to water penetration and rain-proofing has already been recognized by Bartell *et al.* and by Baxter and Cassie in connexion with artificial fabrics. The essential differences may be described under three headings:

(*a*) There are regions where the hairs are wetted on one lateral surface but not on the other. It is obvious without detailed analysis that in a region where the hairs or cylinders are wetted laterally on one side but not on the other there will be a resultant force due to surface tension drawing the hairs together towards the wetted region, this force having a maximum value of 2γ when the wetting liquid forms a thin film between the hairs. This lateral force is usually of a higher order of magnitude than that encountered by plastron hairs when completely submerged (Crisp, 1949) which is a difference term between two similar horizontal forces. Hence, in order to withstand this greater lateral strain, the cylinders or hairs must be more rigidly fixed either by cross-attachments or by increasing their dimensions and in particular their diameter.

(*b*) There is an advantage in a high (apparent) contact angle between the liquid and the external surface which would promote run-off of the liquid. Cassie and Baxter (1944) have shown that in order to promote a high apparent contact angle and quick run-off, it is desirable for a surface to have as open or porous a structure as possible, as the liquid will have then the minimum contact with the solid surface. They show quantitatively that

$$\cos\theta' = f_1 \cos\theta - f_2,$$

where θ is the true contact angle for a plane surface (advancing or receding), θ' the apparent contact angle and f_1 and f_2 the fractional areas of solid liquid and air-liquid interface respectively per unit area of the surface.

(*c*) There is no external pressure imposed, $\Delta p \approx 0$. This removes the necessity to reduce the scale of the structures in order to prevent water penetration, so that large-scale hairs, and large intervening spaces can be tolerated. In practice of course Δp is not quite zero owing to gravity, but is very small so that much larger spaces between the hairs can clearly be allowed.

It follows, therefore, that, apart from the desirability in both instances of a high absolute contact angle, the requirements for resistance to penetration under pressure and resistance to application of water at zero pressure run counter to one another. Resistance to penetration requires a fine-scale structure of some rigidity with the maximum solid-liquid contact (especially when $\theta > 90°$) while rain proofing requires a more rigid structure of larger scale dimensions having the minimum solid-liquid contact.

Thus the large stiff hair piles of *Donacia*, the widely spaced scales of *Stenopelmus* and other surface-dwelling weevils, and the interlocked barbs of ducks' feathers are examples of structures well adapted for 'rain proofing', while the very minute hair piles of *Aphelocheirus*, *Stenelmis* and *Cylloepus* show the opposite adaptation for protection against water penetration. We recognize that the somewhat incompatible requirements for these two purposes is an argument against our thesis that the one may have evolved from the other.

SUMMARY

1. The only important examples of plastron respiration outside the Hemiptera (*Aphelocheirus*) are all to be found in the Coleoptera where it has been known or suspected in three groups: (I) the Dryopoid family Elmidae; (II) the Donaciine (Chrysomelid) genus *Haemonia* (*Macroplea*); and (III) *Phytobius* and possibly certain other genera of the Curculionidae (Rhynchophora). It is the object of the present paper to give as comprehensive an account as possible of the extremely interesting series of examples which the order Coleoptera thus provides.

2. *The Dryopoidea.* (*a*) *Habits.* The two standard species used for the basic experimental work on this group were *Elmis maugei* Bedel and *Riolus cupreus* (Mull.). These beetles are found crawling about on submerged stones in swiftly flowing streams and rivers, or other well-oxygenated waters, browsing upon algal growth. They are quite unable to swim and it was established that provided the water is well aerated they need never come to the surface. Elmids may often be seen grooming

their main plastron areas by means of plastron 'brushes' situated on the inner faces of the femora. They may also be seen to capture small oxygen or air bubbles adhering to aquatic plants with their mouthparts and to replenish the gas on their plastron surfaces by pushing and smearing these bubbles over themselves with these same brushes. These two types of behaviour are known as 'plastron replacement activities'. The importance of grooming in keeping the flexible hairs regularly spaced is emphasized.

(*b*) *Plastron.* The plastron area of *Elmis* and *Riolus* is described and figured. The basic respiratory rate of *Elmis maugei* is $1{\cdot}17 \times 10^{-7}$ c.c. O_2/sec. There are eight pairs of open spiracles (Th 2 + Ab 6) each with its closing apparatus. They open into the sub-elytral space which communicates with the plastron area via the lateral groove of articulation of the elytra. This groove is very effectively protected by hydrofuge hairs and constitutes a satisfactory watertight junction. The tracheal system has no air sacs.

(*c*) *Hydrostatic control. Elmis* cannot swim actively but possesses an elaborate method for hydrostatic control which is described and figured in detail. It depends for its efficacy on proper functioning of the plastron. Both *Elmis* and *Riolus* can control buoyancy so successfully that they can float or sink at will, rising and falling in the water in rapid succession if necessary.

(*d*) *Respiratory efficiency of the Elmid plastron.* Whereas in *Aphelocheirus* the volume of the plastron is extremely constant owing to the erect and rigid plastron hairs, in Elmids, after a bubble has been captured and smeared over the plastron as described above, the hairs (owing to their flexibility and degree of overlap) become slightly more erect and fluffed out. The plastron area is then correspondingly thicker and the sheen more brilliant. This enhanced or thickened layer of gas we distinguish from the more tenaciously held and duller sheen (plastron) by the term 'macroplastron'. This thicker layer of gas is unstable and is actively maintained by the 'plastron replacement activities' of the insect. It thus constitutes a first line of defence against unfavourable environmental conditions. When such conditions supervene and opportunities for plastron replacement from bubbles no longer occur, the macroplastron is lost, the hairs pack down more closely leaving the plastron proper which is held more tenaciously and does not require replacement under ordinary conditions. The plastron of *Elmis* is in every respect inferior to that of *Aphelocheirus*. *Elmis*, therefore, although a true plastron insect, has nothing like the latter insect's margin of safety against wetting under increased pressure and in fact is only able to withstand a pressure difference of slightly less than 1 atm. Once the macroplastron is lost the more closely packed hairs tend to occlude the interface obstructing the diffusion paths with a resulting decrease in respiratory efficiency.

The Dryopoidea can be divided into three fairly distinct groups on the basis of hair-pile dimensions and waterproofing efficiency.

3. *Haemonia* (*Macroplea*). As far as is known this is the sole Donaciine genus which carries a plastron and is thus independent of visits to the surface.

(*a*) The Plastron in *Haemonia* covers almost the whole of the ventral surface as well as the whole of the long antennae. It is very uniform and even and the hairs are

very stiff with a beautifully adjusted bend of about 130° at the tip giving an extremely smooth plastron interface without the excessive overlapping and consequent tendency to pack and occlude the interface which is characteristic of *Elmis* and *Riolus*.

(*b*) *Resistance to wetting.* The plastron is remarkably efficient as a water-protecting mechanism owing to the evenness, rigidity and absence of packing of the hairs. Thus there is need for neither macroplastron, buoyancy control nor plastron replacement activities and all these are in fact absent.

(*c*) *Respiration. Haemonia* shows little immediate response to oxygen lack though it tends to climb upwards when the aquarium is not well aerated and may occasionally be seen with its antennae floating on the surface of the water when they probably have a respiratory function. *Haemonia* can survive severe oxygen lack for periods of several hours.

The tracheal system presents no unusual features and the spiracles are normal save for a highly efficient water-protecting mechanism.

(*d*) *Evolution of plastron respiration in the Donaciinae.* The probable course of evolution of the plastron mechanism of *Haemonia* has been considered by comparison between this genus and typical members of the genus *Donacia*, where the probable function of the hair pile is to protect from wetting during accidental and temporary submergence. Reduction in size and increase in regularity and density of the hairs together with some change in hair shape would be the main steps required to equip *Donacia* for plastron respiration.

4. Of a number of aquatic and semi-aquatic weevils studied *Phytobius velatus* is the only one fully adapted as a plastron insect. This species swims actively and apparently need never come to the surface unless under conditions of very prolonged and severe oxygen deficiency. It carries no air store though there is a small subelytral space and the insect is able to fly. *Phytobius* possesses a complete and highly efficient plastron of minute hairs at a density of $1{\cdot}8–2{\cdot}0 \times 10^8$ per cm.^{2} borne on an almost complete armour or vestiture of touching or overlapping flattened scales. The hairs are parallel to the long axis of the scale and are gently curved at the tip so as to lie along the surface—a plastron arrangement not far from the ideal. In its resistance to wetting the insect shows the same high order of efficiency as *Aphelocheirus*. Average oxygen consumption was found to be 1·24 mm.^{3}/hr. There are no plastron replacement activities and the ability to control buoyancy is very slight. The ability to endure temporary oxygen lack is very high.

5. *General conclusions.* Three important factors in the ability of a hair pile to resist water penetration are discussed. These are: (1) arrangement and regularity; (2) rigidity; (3) scale of hairs.

Aquatic insects which have hydrofuge hairs may be conveniently grouped in four categories the first three of which correspond to those already described in connexion with the Dryopoid beetles.

Series I. Members of this group have a density of 10^8 hairs per cm.^{2} and can probably all withstand a pressure of 2 atm. without wetting. The plastron is very thin and does not afford any reserve of oxygen but functions as a gill. This group comprises Group I of the Dryopoidea, *Phytobius* and *Aphelocheirus*. The plastron of

this group is virtually 'perfect' both structurally and functionally and does not require replacement.

Series II. Comprises the second group of the Dryopoidea and the genus *Haemonia*. These insects have a density of 10^6–10^8 hairs per cm.^{2} and can withstand a pressure of 0·5–2·0 atm. The plastron is thicker than in the previous group but is not sufficient to encumber the insect on account of its buoyancy nor is it sufficient to offer more than a small reserve of oxygen, even when, as in the Elmidae, it is expanded into a 'macroplastron' and actively maintained. The insects in both of these groups have a *functionally* perfect plastron in that they do not need to come to the surface, but that of the Elmids in Group II is not *structurally* perfect and needs maintenance by the activities of the insect.

Series III. This includes the Dryopoid Group III and the Hydrophilids, *Hydrophilus*, *Hydraena* and *Berosus spinosus*. The penetration pressure is less than 0·5 atm. and there are only 10^5–10^6 hairs per cm.^{2}. The plastron in these insects is of considerable volume and acts as much as an air store as it does a gill. It needs periodical renewal at the surface and its volume is sufficient to increase buoyancy to the point at which the insects cannot remain submerged unless either actively swimming or holding on to stones or vegetation. Many of the forms in this group have a double hair pile; the outer set of hairs being readily pressed down on the inner giving them a more complete water protection. The macroplastron formed is thus a definite structure and not a mere increase in the amount of gas in the plastron as it is in the Elmids. It is of course unstable and will be lost by the 'Ege effect' if the insect remains submerged. This series contains insects ranging from almost completely aquatic forms to those which only occasionally enter the water. Periodical grooming behaviour of *Hydrophilus* is described in detail.

Series IV. These insects are without a regular plastron. The hairs only offer protection against accidental or temporary wetting. Many insects living in the proximity of water might be placed in this group with *Donacia*, *Stenopelmus* and *Tanysphyrus*. The hair pile in these forms is more akin to 'rain proofing' than to protection from penetration. The distinction between these two types of protection is discussed.

In all the coleopterous groups investigated it appears that the fully adapted plastron-bearing insect can have been evolved from a riparian form with a hair pile the sole function of which was to enable the insect to enter the water for oviposition or to safeguard it against accidental immersion.

Once again we owe a great debt of gratitude to Mr E. A. Ellis, Naturalist at the Castle Museum, Norwich, for help in obtaining living material. It was through Mr Ellis that we learned of a good locality for *Haemonia mutica* and he and Mr David Langridge were of great assistance in obtaining adequate supplies. Mr E. W. Aubrook of the Tolson Memorial Museum, Ravensknowle, Huddersfield, sent a valuable consignment of *H. appendiculata* from the River Wharfe. To Dr H. E. Hinton of the British Museum (Natural History) we are greatly indebted for a

valuable series of dried specimens of Dryopoidea from which it was possible to estimate the range of respiratory adaptation within this very remarkable group. The material of *Tanysphyrus* and *Stenopelmus* was obtained with the assistance of Dr B. M. Hobby of the Hope Department of Entomology, Oxford, and Dr A. M. Massee also supplied valuable information about localities for other aquatic weevils and himself put much time and effort in the attempt to collect material of these very elusive species for us. Finally, we wish to thank the Rev C. E. Tottenham for frequent help in identification of Hydrophilidae and Rhynchophora.

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EXPLANATION OF PLATE 7

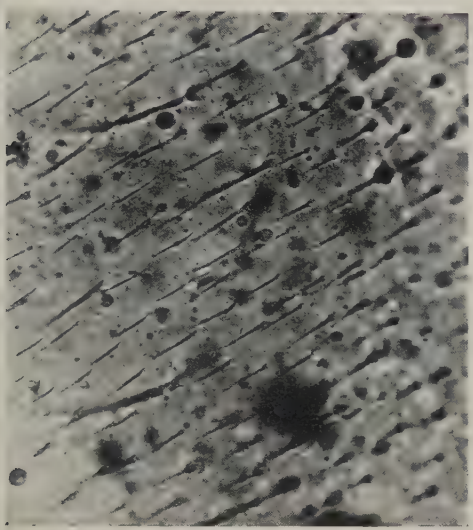
Photomicrographs of gelatin-glycerol replicas of various plastron surfaces.

Fig. *a*. *Hydrophilus piceus* microplastron. ×210.

Fig. *b*. *Elmis maugei* abdominal plastron. ×750.

Fig. *c*. *Macrelmis corisors* abdominal plastron. ×180.

Fig. *d*. *Stenopelmus rufinasus* abdominal plastron scales to show incomplete plastron. The body surface between the scales has been wetted by the replica fluid while many of the scales with their attached plastron hairs are still dry, the meniscus being visible as a sharply defined curved line. ×750.



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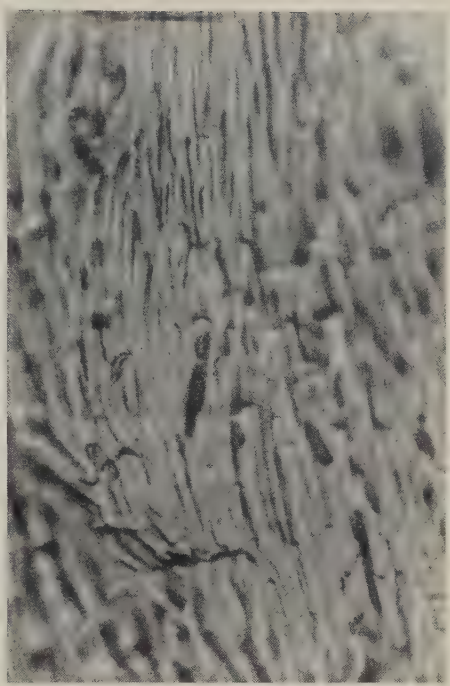

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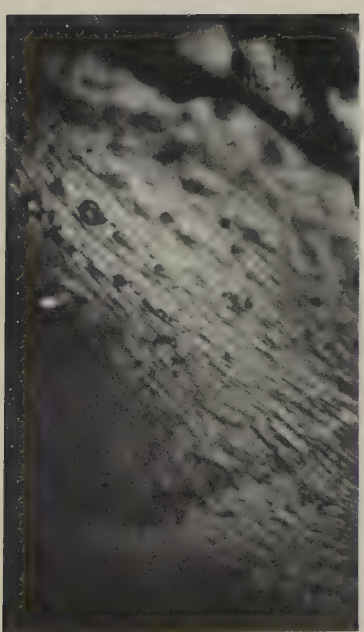

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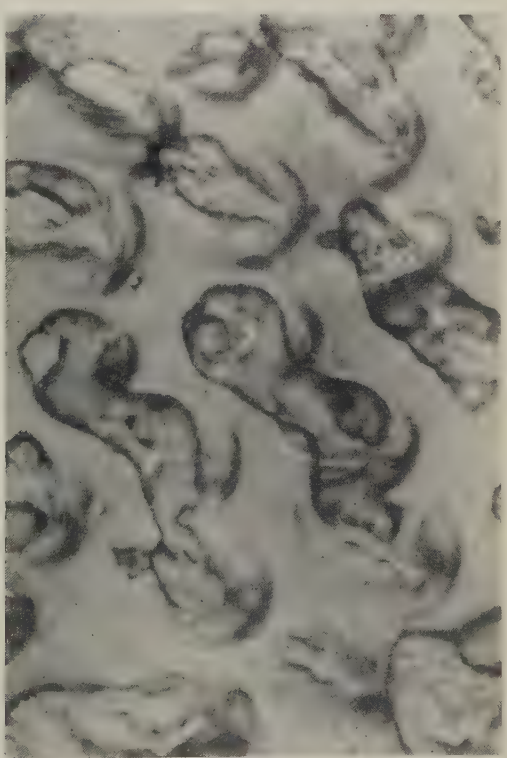

d

THORPE AND CRISP—STUDIES ON PLASTRON RESPIRATION

AGE AND MITOTIC ACTIVITY IN THE MALE MOUSE, *MUS MUSCULUS* L.

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(With Six Text-figures)

I. INTRODUCTION

In earlier analyses of mitotic activity in the male mouse (Bullough, 1948*a*, *b*) it was shown that the routine of waking and sleeping determines the form of the diurnal mitosis cycle, and that changes from the normal in this routine result immediately in changes from the normal in the mitosis cycle. The opinion was then expressed that factors such as the age and sex of the animals, and the habits of the laboratory staff, might be expected to influence the daily round of exercise and rest, and so to affect the form of the mitosis cycle.

Opportunity has now been found to discover the effect of age on the daily routine and mitotic activity of the mouse, and the following paper is a review of conditions in the male.

II. MATERIAL AND METHODS

(1) *The mice*

As in previous work, the observations were all made on mice of two pure line strains, Kreyberg's white label albinos and Strong's *CBA* agoutis. In the younger mice no differences were found between the strains, but with increasing age marked differences developed in both habits and mitotic activity. It is therefore unfortunate that so many of the mice of the older age groups were of one strain, the Strong's *CBA* agouti. This was due to the fact that these males rarely fight, and are therefore far more easily kept for long periods than are the more irritable Kreyberg's mice.

The results took about 12 months to collect, and thus represent mice examined at all seasons of the year. However, apart from unavoidable variations in the length of day, conditions were kept as uniform as possible. The room temperature was maintained at 20° C., and the mice received a regular diet of rat cake, dog biscuit, oats or flaked maize, and chopped carrots. Invariably they were given their food between 09.00 and 10.00 hr., and always it was given in excess so that at no time were they without something to eat.

(2) *The times of day*

The times of day recorded in the various experiments sometimes represent Greenwich mean time, sometimes British summer time, and occasionally double British summer time. In practice it was found unnecessary to record which of these systems was in operation when an experiment was performed, since the animals quickly adapted themselves to a change of the clock. It thus became abundantly

clear that the daily habits of the animals were adjusted to the time of feeding, and they took only a few days to become accustomed to an hour's change either way.

(3) *The earclip technique*

This has already been described in detail by Bullough (1948*a*). Small pieces of ear were removed at intervals by means of a conchotome, and were fixed in Bouin's alcoholic fluid. After sectioning at a thickness of 7 μ, the mitoses were counted in section lengths of 1 cm. From each earclip ten such counts were made, and from these an average figure was obtained. As each experimental group usually consisted of clips from ten mice, ten average figures were available from which to derive the mean and standard error. The latter was calculated by the method for small samples recommended by Simpson & Roe (1939).

(4) *The colchicine technique*

As a check on the results obtained by the earclip technique, mice were killed and examined after their mitoses had been arrested by means of colchicine. To each adult animal, weighing about 25 g., 0·1 mg. of colchicine dissolved in 0·25 c.c. of water was injected subcutaneously, but juvenile animals received proportionately less according to their weight. After 12 hr. the mice were chloroformed, dissected widely open, and fixed whole in Bouin's alcoholic fluid.

Half of each group of animals was injected at 09.00 hr. and killed at 21.00 hr., while half was injected at 21.00 hr. and killed at 09.00 hr. Thus it was planned that a complete period of 24 hr. should be covered by each experiment. However, after the experiments had been completed and the stock of mice used up, it became evident that colchicine not only arrests mitosis in the metaphase, but that it also depresses the number of resting cells which enter the prophase. A separate investigation of this point was then made (Bullough, 1949*b*), and as a result it had to be concluded that the colchicine experiments as recorded here do not reveal as much as was hoped of the degrees of mitotic activity typical of the different age groups.

(5) *Spontaneous bodily activity*

For comparison with the differences observed in the mitotic activity of the different age groups, the spontaneous bodily activity of the mice was also studied. For this purpose, five mice at a time were kept in a box containing two compartments connected by a small hole. In the hole was a hinged door which was pushed aside each time an animal passed, the movement being communicated to a spring arm and recorded on a revolving smoked drum.

Each group of mice remained in the apparatus for 20 days at a time, so that for each hour of the day and night twenty figures were obtained representing the spontaneous activity of the five mice. From these twenty sets of figures, averages and standard errors were calculated.

III. OBSERVATIONS

(1) *Monthly analyses of epidermal mitotic activity*

A study was first made of the mitosis cycles of normal male mice during each of the first 20 months of life. At the age of 20 months mice can be considered old, although there are great variations in this respect between different strains. Thus 20-month-old Strong's *CBA* mice are usually in good condition and, if carefully tended, they may live for another year or more, while 20-month-old Kreyberg's white label mice are usually thin and feeble and, in the best of conditions, they have only a short expectation of life. Difference in what has been termed the physiological age may also be induced by the conditions in which the mice live during their first 20 months, and by the diseases which they may have. In the experiments recorded here all the mice were subjected to the same conditions, and all were free from disease.

It appeared that the most satisfactory way to study the mitotic activity in each month of life would be to follow the cycle through a complete period of 24 hr., and then to repeat the experiment at a later date as a check on the first results. However, the effort required, and especially the numbers of mice needed, proved too great for this to be done. Consequently only those variations occurring between 08.00 and 20.00 hr. were analysed. This 12 hr. interval covered the feeding period between 09.00 and 10.00 hr. when the mice were always disturbed, the early afternoon sleep period during which the animal room was always quiet with all age groups resting, and the evening period of activity in which, by 20.00 hr., all animals were observed to be fully awake once more. Thus the interval 08.00–20.00 hr. could be expected to begin and end with periods of low mitotic activity, and to contain somewhere within it a period of high mitotic activity. During this time seven earclips were taken at 2 hr. intervals, and, since all of these could easily be obtained from one ear, it was possible to conserve the other ear for use in a subsequent month.

Whenever possible the results for each month were confirmed by a second experiment performed at a different time with different mice. Because of the limited supply this was not always possible, but it was usually found that confirmation of the results for any one month was provided by the results for the months which preceded and followed it.

It quickly became obvious that striking changes in the mitotic activity of the ear epidermis do occur with advancing age, and it was possible to distinguish four types of cycle which differed from one another in amplitude and often also in timing. These four ages of the mouse can be named as follows:

The immature age	From 1 to 3 months
The mature age	From 3 to 12 months
The middle age	From 13 to 18 months or later
The senile age	From the end of the middle age to death

The differences in mitotic activity between these various ages are clear cut, and an analysis of them is given below.

The immature age

In this period of life the animals are still actively growing, and they range in weight from under 10 g. to just over 20 g. Sections of the testes showed that at 1 month spermatozoa were forming but had not yet been released into the epididymis. By 2 months the epididymis was full of spermatozoa, and the animals were presumably in breeding condition. When given the opportunity, mice of these strains have been known to breed at an age of 5 or 6 weeks.

The characteristics of the epidermal mitotic activity of these young animals are indicated in the following Table 1.

Table 1. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten immature male mice*

Time of day	Strong's *CBA* mice aged 1 month	Strong's *CBA* mice aged 2 months	Kreyberg's white label mice aged 2 months	Strong's *CBA* mice aged 3 months
08.00	3·7 ± 0·19	4·0 ± 0·28	3·9 ± 0·26	3·1 ± 0.21
10.00	2·4 ± 0·17	2·7 ± 0·43	1·8 ± 0·15	2·7 ± 0·19
12.00	4·3 ± 0·55	4·1 ± 0·37	4·0 ± 0·23	3·9 ± 0·31
14.00	5·3 ± 0·51	5·0 ± 0·20	5·5 ± 0·21	5·0 ± 0·14
16.00	1·7 ± 0·17	3·6 ± 0·25	3·1 ± 0·27	3·5 ± 0·22
18.00	2·1 ± 0·23	1·0 ± 0·14	1·4 ± 0·12	1·4 ± 0·18
20.00	2·0 ± 0·27	2·1 ± 0·17	2·7 ± 0·14	1·2 ± 0·13
Totals	21·5	22·5	22·4	20·8

Important points to notice in this table are that the peak of mitotic activity was at 14.00 hr., and that the highest average number of mitoses observed per unit section length of 1 cm. was only about 5. Further, it will be noticed that the total of the average numbers of mitoses in each group of observations was about 22, and that there were no differences between the Strong's *CBA* males and the Kreyberg's white label males.

The mature age

This age apparently begins quite abruptly when a mouse ceases to grow actively on reaching a weight of about 25 g., the testes then being fully active. This usually seems to happen some time about the beginning of the third month of life, and the first example, given in Table 2 below, is of a group of mice of that age. The mature age continues until the mouse is about a year old, and during that time little or no variation in the mitosis rate was found. The characteristics of the mitotic activity of this age can be seen from the Tables 2 to 4.

A survey of these tables indicates that between the ages of 3 and 12 months the time of maximum mitotic activity commonly remains, as in the immature animals, at about 14.00 hr. However, in these mature animals the maximum number of mitoses per unit section length of 1 cm. has risen from the immature figure of 5 to a new figure of about 8. Similarly, the totals of the average numbers of mitoses observed has risen from the immature figure of about 22 to a new figure of about 30.

Thus the mature age is characterized by an apparent rise in the mitosis rate. The question of whether this increase is real or illusory is dealt with in a later section.

Table 2. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten mature male mice*

Time of day	Kreyberg's white label mice aged 3 months	Kreyberg's white label mice aged 4 months	Strong's *CBA* mice aged 5 months	Kreyberg's white label mice aged 6 months	Strong's *CBA* mice aged 7 months
08.00	5·1 ± 0·21	4·1 ± 0·24	4·2 ± 0·21	3·6 ± 0·29	3·1 ± 0·12
10.00	3·2 ± 0·13	2·5 ± 0·19	3·1 ± 0·29	2·5 ± 0·16	2·1 ± 0·18
12.00	5·4 ± 0·19	6·4 ± 0·42	5·5 ± 0·47	5·6 ± 0·37	4·6 ± 0·28
14.00	7·4 ± 0·32	8·2 ± 0·46	8·9 ± 0·31	9·8 ± 0·47	8·2 ± 0·32
16.00	6·4 ± 0·31	5·5 ± 0·33	5·1 ± 0·26	5·3 ± 0·26	5·6 ± 0·24
18.00	5·9 ± 0·16	2·9 ± 0·15	2·3 ± 0·19	2·8 ± 0·22	3·7 ± 0·13
20.00	1·6 ± 0·10	1·7 ± 0·07	1·2 ± 0·11	1·4 ± 0·15	1·7 ± 0·05
Totals	35·0	31·3	30·3	31·0	29·0

Table 3. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten mature male mice*

Time of day	Strong's *CBA* mice aged 8 months	Kreyberg's white label mice aged 9 months	Strong's *CBA* mice aged 9 months	Kreyberg's white label mice aged 10 months	Strong's *CBA* mice aged 10 months
08.00	4·7 ± 0·23	8·6 ± 0·34	5·2 ± 0·16	5·1 ± 0·18	8·9 ± 0·54
10.00	3·5 ± 0·34	2·0 ± 0·24	2·3 ± 0·14	2·1 ± 0·17	1·6 ± 0·29
12.00	3·0 ± 0·28	1·1 ± 0·16	4·1 ± 0·21	2·2 ± 0·24	3·6 ± 0·32
14.00	7·0 ± 0·41	7·4 ± 0·32	7·5 ± 0·32	7·1 ± 0·30	4·0 ± 0·20
16.00	5·2 ± 0·43	5·0 ± 0·22	5·5 ± 0·28	5·4 ± 0·35	9·2 ± 0·24
18.00	4·2 ± 0·17	3·1 ± 0·24	4·6 ± 0·27	5·2 ± 0·21	4·1 ± 0·27
20.00	2·7 ± 0·20	3·4 ± 0·33	2·9 ± 0·12	2·8 ± 0·11	1·1 ± 0·17
Totals	30·3	30·6	32·1	29·9	32·5

Table 4. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten mature male mice*

Time of day	Kreyberg's white label mice aged 11 months	Strong's *CBA* mice aged 11 months	Kreyberg's white label mice aged 12 months	Strong's *CBA* mice aged 12 months
08.00	5·3 ± 0·33	5·4 ± 0·25	5·5 ± 0·38	4·7 ± 0·22
10.00	2·6 ± 0·16	7·1 ± 0·31	1·7 ± 0·25	3·6 ± 0·17
12.00	3·4 ± 0·18	5·3 ± 0·26	2·9 ± 0·30	7·3 ± 0·36
14.00	7·3 ± 0·26	4·2 ± 0·21	7·0 ± 0·24	5·0 ± 0·31
16.00	4·3 ± 0·27	1·9 ± 0·17	3·9 ± 0·14	4·1 ± 0·18
18.00	3·1 ± 0·15	2·1 ± 0·23	4·0 ± 0·20	2·3 ± 0·18
20.00	2·2 ± 0·14	2·5 ± 0·18	4·2 ± 0·14	1·9 ± 0·13
Totals	28·2	28·5	29·2	28·9

A further point of interest also emerges from these tables. It can be seen that while the Kreyberg's white label mice continued with great regularity to develop maximum mitotic activity at 14.00 hr., the Strong's *CBA* mice did not. While these

latter mice maintained their mitosis rate apparently unchanged, they tended after the age of about 10 months to develop maximum mitotic activity at an earlier hour. As will be seen in the following section, this tendency towards an earlier peak of mitotic activity is continued during middle age in mice of this strain.

The middle age

The next change in the mitosis rate was apparently abrupt, and was observed in 13-month-old Strong's *CBA* mice. About this time the animals seemed to be entering into what, by human analogy, can be called middle age. They usually appeared quieter and lazier, but the only positive sign of metabolic change was their increasing weight. The mature males had a steady average weight of about 25 g., but at 12 or 13 months almost all the animals began to lay down deposits of fat. This process was particularly marked in Strong's *CBA* mice, some of which reached weights of over 50 g. at an age of about 16 months. There was no other external sign of advancing age, and the fur remained glossy and thick. Internally, apart from the fat deposits, there was also no apparent change. The testes remained indistinguishable from those of younger mice, and, with one exception, all the middle-aged males examined showed active spermatogenesis with the epididymes full of spermatozoa. The single exception, a Strong's *CBA* mouse aged 16 months, had two shrivelled testes and lacked any spermatozoa. It was undoubtedly abnormal.

However, if it was difficult to point to any other feature which could be used as a positive sign that the middle-aged condition had been reached, the change in the mitosis rate at this time appeared to be certain and complete. This is brought out in the Tables 5 and 6 for Strong's *CBA* mice from which the characteristics of this middle-age period can be readily perceived.

For an understanding of the peculiarities of mitotic activity in the middle-age period, the figures of the 13- and 14-month-old mice are the most valuable. They show a sudden apparent increase in the mitosis rate, so that the maximum number of mitoses per unit section length of 1 cm. rose from an earlier figure of about 8 to the new figure of about 14. Similarly, the total of the average numbers of mitoses observed during the 12 hr. period rose from the earlier figure of about 30 to the new figure of about 47. The question of whether this increase is real or not is dealt with in a later section.

The other obvious change in the mitosis cycle shown by these tables is the steady shift of the period of maximum mitotic activity from 12.00 to 10.00 hr. and then from 10.00 to 08.00 hr. Further, it was observed that by the age of 16 months almost all the mitoses counted at 08.00 hr. were in the telophase. This was also true in the 17- to 20-month age groups, and it is therefore obvious that in all these cases the period of maximum mitotic activity had passed before the observations were commenced at 08.00 hr. This explains the lower total numbers of mitoses observed in these older groups.

It is also interesting to note that when the main peak of mitotic activity moved forward to 08.00 hr. or earlier, a small secondary burst of activity developed sometime between 12.00 and 16.00 hr. This was perhaps clearer in the sections themselves

than it is in the tables because, while up to 12.00 hr. the mitoses were mainly in the ana- and telophases, in the early afternoon pro- and metaphases predominated. These in turn gave place to telophases by 16.00 and 18.00 hr.

Table 5. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten middle-aged male mice*

Time of day	Strong's *CBA* mice aged 13 months	Strong's *CBA* mice aged 14 months	Strong's *CBA* mice aged 15 months	Strong's *CBA* mice aged 16 months
08.00	4·2±0·25	5·9±0·36	14·9±0·65	14·0±0·22
10.00	9·1±0·51	13·1±0·62	5·6±0·31	5·7±0·23
12.00	14·9±0·57	10·7±0·49	3·8±0·22	3·8±0·20
14.00	9·3±0·32	6·7±0·27	4·7±0·23	4·5±0·18
16.00	4·0±0·27	5·6±0·40	3·5±0·38	2·0±0·16
18.00	2·7±0·19	3·1±0·16	2·2±0·26	0·9±0·14
20.00	2·8±0·18	2·3±0·19	3·1±0·41	2·2±0·18
Totals	47·0	47·4	37·8	33·1

Table 6. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten middle-aged male mice*

Time of day	Strong's *CBA* mice aged 17 months	Strong's *CBA* mice aged 18 months	Strong's *CBA* mice aged 19 months	Strong's *CBA* mice aged 20 months
08.00	13·9±0·43	14·0±0·47	13·9±0·33	12·6±0·45
10·00	6·3±0·31	11·5±0·64	9·4±0·30	5·9±0·37
12.00	4·0±0·23	3·7±0·28	5·4±0·32	2·9±0·39
14.00	4·5±0·25	4·0±0·29	4·6±0·18	5·0±0·41
16.00	5·2±0·31	1·7±0·22	3·2±0·31	6·3±0·49
18.00	1·7±0·12	2·9±0·29	1·2±0·19	6·6±0·35
20·00	2·5±0·15	2·0±0·19	1·6±0·21	1·2±0·24
Totals	38·1	39·8	39·3	40·5

Table 7. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups of middle-aged male mice*

Time of day	Eight Kreyberg's white label mice aged 14 months	Five Kreyberg's white label mice aged 15 months	Seven Kreyberg's white label mice aged 16 months	Ten Kreyberg's white label mice aged 18 months
08.00	5·5±0·19	7·5±0·36	9·4±0·31	6·8±0·36
10.00	3·7±0·23	4·0±0·21	3·6±0·21	9·1±0·48
12.00	14·8±0·44	8·7±0·31	3·7±0·33	12·6±0·45
14.00	7·6±0·28	16·2±0·63	5·5±0·66	9·7±0·53
16.00	7·1±0·43	12·1±0·51	18·2±0·58	5·1±0·30
18.00	4·0±0·24	4·6±0·26	10·8±0·56	4·2±0·31
20.00	3·5±0·22	3·9±0·18	8·9±0·72	3·0±0·24
Totals	46·2	57·0	60·1	50·5

These results for the Strong's *CBA* mice have been described separately since they differ somewhat from those obtained with the Kreyberg's white label mice. These latter results are given in Table 7.

In the first place it may be stressed that these figures for the Kreyberg's mice confirm the fact that an apparent increase in the mitosis rate accompanies the transition to middle age. Compared with the maximum figures of about 8 mitoses obtained for mature mice, the new maximum lies between about 12 and 18. Similarly, the total of the average numbers of mitoses observed during the 12 hr. period of the experiment has risen in middle age from the earlier figure of about 30 to a new figure of between 46 and 60.

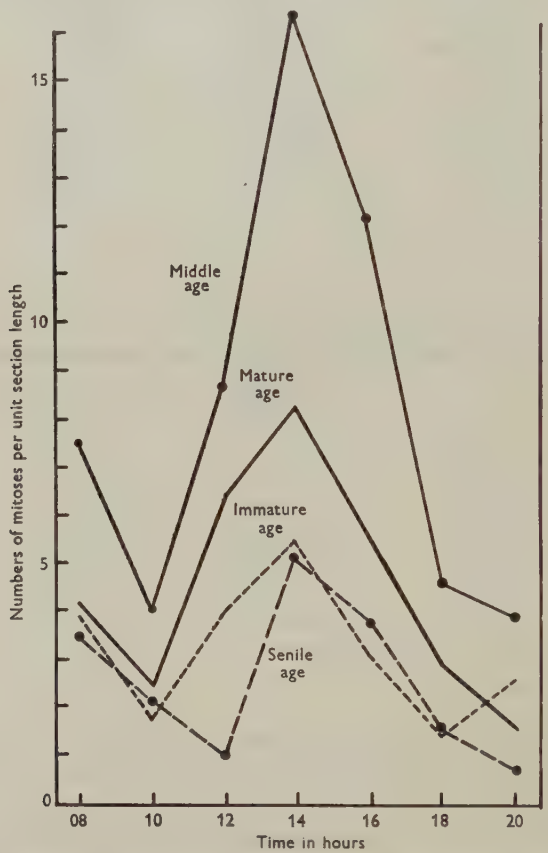

Fig. 1. The diurnal variations in the mitosis activity of the ear epidermis in groups of Kreyberg's white label males of various ages.

However, it will be seen that the Kreyberg's white label mice differed markedly from the Strong's *CBA* mice in that the period of maximum mitotic activity remained at about 14.00 hr. as in younger animals. The timing of this period of maximum mitotic activity was certainly more irregular than in the younger mice, but there is no evidence of any progressive forward shift.

The general conclusions can therefore be reached that the period of middle age, which begins at about 13 months, is characterized by an apparent rise in mitotic activity, and that, according to the strain of mouse used, this change may or may not be accompanied by changes in the timing of the mitosis cycle.

The senile age

Relatively few data are available for the senile age, which is characterized by feebleness and great loss of weight. In the Kreyberg's white label mice these changes were apparent before the age of 20 months, but in the Strong's *CBA* mice they were not. The Kreyberg's mice of 19 and 20 months, on which Table 8 is based, were emaciated with weights of less than 20 g. Their backs were permanently arched, and their fur was thin and poor. They were extremely feeble, and they spent almost all their time lying quietly in a corner.

Table 8. *The variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups of senile male mice*

Time of day	Ten Kreyberg's white label mice aged 19 months	Seven Kreyberg's white label mice aged 20 months
08.00	4·1 ± 0·30	3·5 ± 0·28
10.00	1·7 ± 0·31	2·1 ± 0·13
12.00	5·3 ± 0·27	1·0 ± 0·20
14.00	3·0 ± 0·22	5·1 ± 0·24
16.00	3·6 ± 0·30	3·8 ± 0·26
18.00	1·2 ± 0·14	1·6 ± 0·22
20.00	0·5 ± 0·10	0·7 ± 0·15
Totals	19·4	17·8

In these old animals the mitosis rate apparently fell to a slightly lower level than that seen in the immature males (Table 1). The time of maximum mitotic activity remained at about 14.00 hr., but at that time the average number of mitoses per unit section length of 1 cm. was only about 5 as compared with almost 13 in the 18-month-old Kreyberg's mice. A similar fall is seen in the total of the average numbers of mitoses observed during the 12 hr. period of the experiment. In these senile mice the figure was only about 18 or 19 as compared with about 50 for the 18-month-old animals.

(2) *Analyses of full diurnal cycles*

Following this preliminary survey, the observations were checked and extended by a study of each type of cycle in greater detail. The ages so examined were 2, 6 and 17 months, these being typical respectively of the immature, mature, and middle ages. Each experiment covered a full period of 24 hr., and the earclips were taken at 2 hr. intervals from 08.00 hr. on one day to 08.00 hr. on the next. It is unfortunate that no senile Kreyberg's mice were available for this experiment. Because of this the senile age group was omitted altogether, and the observations were restricted to Strong's *CBA* mice.

The immature age

The results obtained with the 2-month-old males are recorded in Table 9 and shown graphically in Fig. 2. They afford still further confirmation of the results previously obtained with immature males (Table 1). They also indicate that, in the conditions of the experiment, an immature male experiences periods of maximum

mitotic activity at 04.00, 08.00 and 14.00 hr., and periods of minimum mitotic activity at 06.00, 10.00 and 22.00 hr.

It can also be seen that in none of the periods of maximum activity did the average numbers of mitoses present per unit section length of 1 cm. rise above 5·8, while the lowest recorded figure at 10.00 hr. was 1.4. The total of the average numbers of mitoses observed between 08.00 hr. on the first day and 06.00 hr. on the second day was approximately 40.

The mature age

The second group of males, ten Strong's *CBA* mice aged 6 months, gave the figures recorded in Table 9 and in the graph in Fig. 2.

Table 9. *The diurnal variations in the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten male mice*

Time of day	Strong's *CBA* mice aged 3 months	Strong's *CBA* mice aged 6 months	Strong's *CBA* mice aged 17 months
08.00	4·5 ± 0·20	4·3 ± 0·18	15·3 ± 0·49
10.00	1·4 ± 0·13	2·7 ± 0·26	5·1 ± 0·34
12.00	4·8 ± 0·26	5·1 ± 0·34	4·4 ± 0·19
14.00	5·8 ± 0·18	8·6 ± 0·40	6·1 ± 0·16
16.00	3·2 ± 0·13	6·8 ± 0·24	5·0 ± 0·31
18.00	2·7 ± 0·16	5·7 ± 0·25	2·7 ± 0·18
20.00	2·8 ± 0·25	2·2 ± 0·11	2·6 ± 0·28
22.00	1·5 ± 0·17	3·9 ± 0·15	4·7 ± 0·22
24.00	2·3 ± 0·22	4·5 ± 0·12	13·5 ± 0·30
02.00	3·6 ± 0·19	4·9 ± 0·19	9·0 ± 0·39
04.00	5·6 ± 0·26	5·1 ± 0·32	3·6 ± 0·22
06.00	1·6 ± 0·13	9·1 ± 0·37	8·6 ± 0·48
08.00	4·2 ± 0·26	5·5 ± 0·23	14·8 ± 0·35
Totals	44·0	68·4	95·4

These results are in confirmation of those recorded above for mature males (Tables 2–4). Compared with the immature animals, there were only two periods of maximum mitotic activity at 06.00 and 14.00 hr., and two periods of minimum activity at 10.00 and 20.00 hr., and hour by hour the mitosis rate was on a higher level. The greatest average number of mitoses present per unit section length of 1 cm. was 9·1 and the lowest was 2·2. These figures can be compared with 5·8 and 1·4 respectively in the immature animals. As a final point of contrast, it should be noted that the total of the average numbers of mitoses observed between 08.00 hr. on the first day and 06.00 hr. on the second was about 63, which represents a rise of approximately 55% over the figure for the immature animals.

The middle age

Table 9 and Fig. 2 also include an analysis of the mitosis counts from the group of ten Strong's *CBA* males aged 17 months. Once again confirmation is provided for the results given earlier (Tables 5 and 6), and it is very clear that the diurnal mitosis cycle of these older males differs from that of the younger males both in its timing and

in its amplitude. The main periods of maximum mitotic activity were at 08.00 and 24.00 hr., and there was a lesser burst of activity at 14.00 hr. on which comment has already been made. The maximum average number of mitoses present per unit section length of 1 cm. reached the high level of 15·3, while the minimum number fell no lower than 2·6. The total of the average numbers of mitoses observed between 08.00 hr. on the first day and 06.00 hr. on the second was about 80, which is an increase of about 30% over the figure for the mature males and of about 100% over the figure for the immature males.

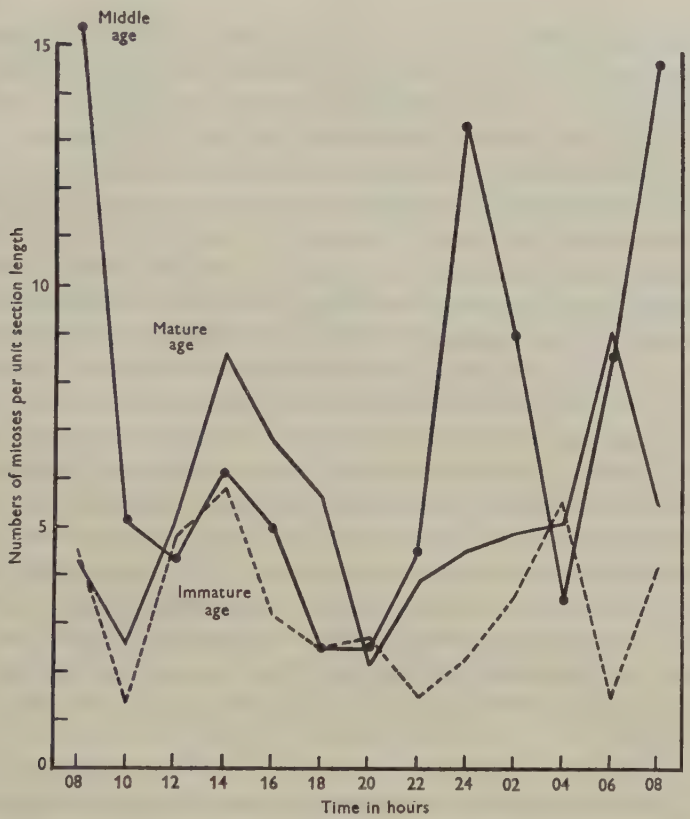

Fig. 2. The diurnal variations in the mitotic activity of the ear epidermis in groups of Strong's *CBA* males of various ages.

(3) *Experiments with colchicine*

From all the experiments recorded above it can safely be concluded that the diurnal cycles of epidermal mitotic activity in Strong's *CBA* males differ in timing in the immature, mature and middle ages, while in Kreyberg's white label males the timing of the cycles of mature, middle-aged, and senile animals is essentially the same.

However, the most important point still remains obscure. This is the question of whether in fact the ear epidermis of the immature male has a lower mitosis rate than that of the mature male, and whether this in turn has a lower mitosis rate than that

of the middle-aged male. While all the evidence suggests that this is so, the conclusion is so unexpected that other explanations must be sought. The most obvious alternative possibility is that the results obtained may be due to age changes in the speed at which each division is completed. If in a mature animal the speed of completion of a mitosis is less than in an immature animal, then it might be expected that the former would show more mitoses at any given moment than would the latter. Again, if the speed of completion of a division is still further reduced in middle age, then the numbers of mitoses visible at any given moment might be expected to rise once more.

However, in the case of the senile animal it can hardly be supposed that a sufficient increase in the speed of division could occur to account for the fall in the numbers of mitoses observed, and it appears reasonable to draw the immediate conclusion that this age is in fact characterized by a real reduction in the mitosis rate.

An attempt to answer this whole question was made with the use of colchicine, which is considered to arrest all mitoses at about the metaphase. The theory underlying these experiments was that by means of a single injection all the mitoses beginning during the subsequent 12 hr. period could be arrested, so that, after sectioning and counting in the manner already described, a fairly accurate estimate could be obtained of the numbers of mitoses which normally occur during this period. In this way any complication due to the speed of completion of the divisions could be eliminated, and, with two experiments, the full period of 24 hr. could be covered and an estimate made of the total number of mitoses which occur daily.

In practice, however, difficulties were encountered which are best discussed after a consideration of the results. These difficulties made it necessary to consider the Kreyberg's mice separately from the Strong's mice, as is done in Tables 10 and 11. The mice concerned were injected with colchicine at 09.00 hr. and killed at 21.00 hr. to cover the 12 hr. of day, or injected at 21.00 hr. and killed at 09.00 hr. to cover the 12 hr. of night. Each adult mouse received 0·1 mg. of colchicine dissolved in 0·25 c.c. of water which was injected subcutaneously. Each immature mouse, being approximately half the weight of an adult, received only 0·05 mg. of colchicine in 0·125 c.c. of water.

Table 10. *The average numbers of mitoses arrested by colchicine in 12 hr. in unit section lengths (1 cm.) of the ear epidermis in groups each of ten Kreyberg's white label males*

Age of mice	Period 09.00–21.00 hr.	Period 21.00–09.00 hr.
1 month	4·8 ± 0·16	4·1 ± 0·21
5 months	10·3 ± 0·48	5·7 ± 0·32
16 months	15·4 ± 0·55	12·8 ± 0·71

From Table 10 it would appear that the total numbers of mitoses occurring in a period of 24 hr. in each cm. length of sections of ear epidermis cut 7 μ thick was in immature males about 9, in mature males about 16, and in middle-aged males

about 28. While this affords strong confirmation that real increases in the mitosis rate do occur with increasing age, all these figures are considerably smaller than would be expected from the other tables given above. Thus a doubt immediately arises as to the accuracy of the method, and this doubt is increased by a consideration of the results from the Strong's *CBA* mice given in Table 11.

Table 11. *The average number of mitoses arrested by colchicine in 12 hr. in unit section lengths (1 cm.) of the ear epidermis in groups each of ten Strong's CBA males*

Age of mice	Period 09.00–21.00 hr.	Period 21.00–09.00 hr.
1 month	3·7 ± 0·31	4·5 ± 0·26
4 months	12·2 ± 0·67	6·8 ± 0·33
6 months	10·8 ± 0·61	6·0 ± 0·35
14 months	9·4 ± 0·73	7·6 ± 0·52
15 months	14·7 ± 0·81	5·6 ± 0·22
16 months	11·7 ± 0·54	5·5 ± 0·38
20 months	13·0 ± 0·62	8·5 ± 0·48

From this table it is evident that the results for the immature males, with a daily mitosis total of about 8, and for the mature males, with a daily mitosis total of about 18, are similar to those obtained with the Kreyberg's mice. The difference is seen in the middle-aged males, which in this table show little or no increase over the mitosis rate typical of the mature animals. The daily mitosis totals of the middle-aged Strong's males lie between about 17 and 21, and so do not approach the middle-aged Kreyberg's males' figure of 28.

From all these colchicine results a strong suspicion arises that the drug not only arrests mitosis in the metaphase, but that it also slows down the rate at which the resting cells enter the prophase. If this is so, then the results obtained cannot be regarded as an indication of normal conditions, and in order to clear up this point it was necessary to carry out an investigation into the action of colchicine using the earclip technique (Bullough, 1949*b*). This investigation showed that only for a period of about 5 hr. after the injection of 0·1 mg. of colchicine does the epidermal mitosis rate remain normal. After 5 hr. a depressing effect rapidly develops, and after 6 hr. mitosis stops altogether. Thus, in the colchicine experiments recorded in Tables 10 and 11, the mitoses observed were those which developed during only the first 5 hr. of the 12 hr. period.

With this in mind, the results can be interpreted as follows. In all the three age groups of Kreyberg's mice injected at 09.00 hr., the colchicine must have arrested in or about the metaphase those mitoses which developed during the period up to 14.00 hr., the usual time of maximum mitotic activity associated with the afternoon sleep period. Thus these three sets of figures are strictly comparable, and it can be concluded that the numbers of mitoses which are involved in the rise to maximum activity in immature, mature, and middle-aged animals are in the approximate proportion of 1:2:3.

In the same way, the figures for the immature and mature Strong's *CBA* males injected at 09.00 hr. are also comparable, and are in the same approximate propor-

tion of 1:2. It is the figures for the middle-aged Strong's males which cannot be compared, since it is evident from Tables 5, 6 and 9 that in this strain the period 09.00–14.00 hr. is not a time of greatly increasing mitotic activity. Instead, the maximum activity is passed before 08.00 hr., and the increased activity which has been noted at about 14.00 hr. is slight by comparison.

Of the figures obtained for the period 21.00–09.00 hr., little can be said except that in general they support the evidence provided by the figures for the period 09.00–21.00 hr. It is important to bear in mind that the mitosis cycle is less regular during the night, probably because of the lack of a feeding time by which it can be stabilized.

While admitting the generally unsatisfactory nature of these colchicine experiments, it is legitimate to conclude that they offer the strongest evidence that a real increase in the mitosis rate of the ear epidermis occurs between the immature and mature ages, and again between the mature and middle ages. As stated earlier, it is also reasonable to conclude that the senile age is characterized by a reduction in mitotic activity to a level slightly below that of the immature mice. It is not yet possible to state accurately the sizes of these increases and decreases, but, judging from all the figures available, they are perhaps in the approximate proportions of immature, 1; mature, 2; middle age, 3; senile, 1.

(4) *Analyses of spontaneous bodily activity*

An attempt was also made to account for the observed changes in the timing of the diurnal mitosis cycles in the different age groups. Since these changes in timing were most pronounced in the Strong's *CBA* strain, all the experiments were performed with these animals.

In view of earlier results (Bullough, 1948*a*, *b*), which related the diurnal changes in the mitosis rate to the periods of waking and sleeping, it was immediately suspected that the age changes in the diurnal mitosis cycles were merely reflexions of age changes in the animal's daily habits. A study was therefore made of activity and rest in immature, mature, and middle-aged mice. All the experiments were conducted in the same way. In each case five males were put into a box with two compartments connected by a small hole, and, by means of a recording device, it was possible to discover the number of times which the five animals passed through the hole in each hour of the day and night. In each experiment the five animals remained in the box for 20 consecutive days, so that twenty sets of figures were obtained from which the averages and standard errors were calculated.

Immature age

The five males used were 4 weeks old at the beginning of the experiment, and 7 weeks old at the end, and the results which they gave are expressed in Table 12 and in Fig. 3. In the figure the results are represented for convenience by a line graph, instead of more correctly by a block graph, and superimposed is the graph of the diurnal mitosis cycle of immature Strong's *CBA* males (Table 9). It is evident that these young animals were never still for long, so that even in the quietest hour of

the day there was an average of about eleven passages through the hole. However, it is clear from the graphs that the quieter periods at about 04.00, 08.00 and 14.00 hr. were also the times of maximum mitotic activity, while the periods of greatest bodily activity at about 06.00, 10.00 and 21.00 hr. were the times of minimum mitotic activity. Of these periods of greatest bodily activity, that at about 10.00 hr. coincided with the daily feeding time, while those at 06.00 and 21.00 hr. approximately coincided with dawn and dusk respectively.

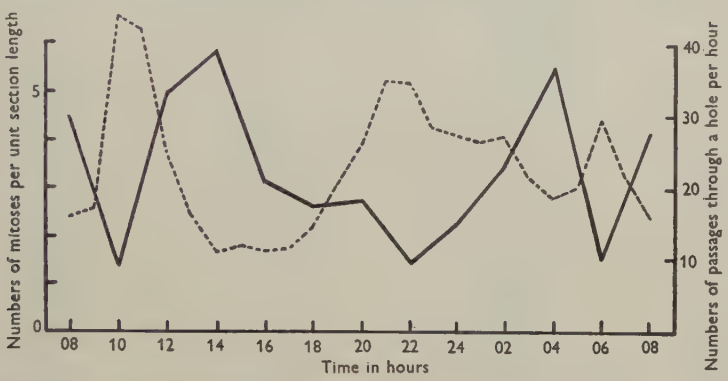

Fig. 3. The diurnal variations in the mitotic activity of the ear epidermis (solid line) as compared with the diurnal variations in the spontaneous bodily activity (broken line) of immature Strong's *CBA* males.

For later comparisons it should also be noted that the total of the average numbers of times which the five animals passed through the hole each day was approximately 565.

Mature age

The Strong's males examined in this experiment were 5 months old, and the results are given in Table 12 and in Fig. 4.

Like the immature animals, these males become extremely active at about 10.00 hr., when they were in the habit of being fed, and were inactive at 14.00 and 15.00 hr., when they observed an afternoon rest period. They became active again in the evening, and they maintained a moderate degree of activity throughout the night. However, unlike the immature males, they did not show any increased activity about dawn, but instead they observed several hours of rest until feeding time.

These points are illustrated in Fig. 4 to which, for comparison, a graph of mitotic activity has been added (Table 9). Once again it is obvious that the times of high bodily activity are also the times of low mitotic activity, while the times of low bodily activity are the times of high mitotic activity.

It should also be noticed that the mature males indulge in less spontaneous bodily activity than do the immature males. Their rest periods are longer and more pronounced, and the total of the average numbers of times which they passed through the hole each day was only about 460 as compared with about 565 for the younger animals.

Table 12. *The spontaneous bodily activity of groups of five male mice expressed as the average numbers of passages through a hole per hour*

Hour ending	Strong's *CBA* mice aged 1 month	Strong's *CBA* mice aged 5 months	Strong's *CBA* mice aged 17 months
09.00	16·6 ± 1·88	10·9 ± 1·35	4·2 ± 1·03
10.00	46·4 ± 3·02	49·6 ± 5·11	5·3 ± 1·21
11.00	43·9 ± 3·00	44·7 ± 3·27	11·8 ± 1·77
12.00	25·0 ± 2·41	24·9 ± 2·70	5·1 ± 1·44
13.00	15·8 ± 2·11	13·4 ± 2·78	4·6 ± 1·10
14.00	11·1 ± 1·86	7·1 ± 1·83	4·4 ± 1·00
15.00	12·2 ± 1·73	5·0 ± 1·08	3·4 ± 0·71
16.00	11·5 ± 0·87	8·5 ± 1·52	2·4 ± 0·62
17.00	11·8 ± 1·43	14·8 ± 2·16	3·5 ± 0·53
18.00	14·5 ± 1·35	26·2 ± 3·52	13·3 ± 1·91
19.00	20·6 ± 1·52	30·1 ± 2·97	23·9 ± 2·32
20.00	27·6 ± 1·27	29·6 ± 2·60	21·8 ± 1·79
21.00	35·0 ± 2·93	24·9 ± 3·16	19·5 ± 1·93
22.00	34·5 ± 3·09	21·3 ± 2·55	15·5 ± 1·62
23.00	28·7 ± 2·38	18·4 ± 2·13	15·0 ± 1·59
24.00	27·0 ± 2·77	21·5 ± 2·59	13·9 ± 1·97
01.00	26·1 ± 1·84	16·8 ± 1·44	16·3 ± 2·98
02.00	27·3 ± 1·92	18·1 ± 1·10	15·1 ± 3·30
03.00	22·4 ± 2·10	17·3 ± 2·12	22·4 ± 2·95
04.00	19·4 ± 1·73	17·0 ± 1·02	16·8 ± 2·70
05.00	20·7 ± 2·04	14·8 ± 2·31	13·3 ± 0·97
06.00	29·4 ± 2·34	10·2 ± 1·17	14·2 ± 5·00
07.00	21·1 ± 1·51	6·3 ± 0·85	8·2 ± 2·78
08.00	15·8 ± 2·76	8·9 ± 1·09	4·0 ± 0·85
Totals	564·4	460·3	277·9

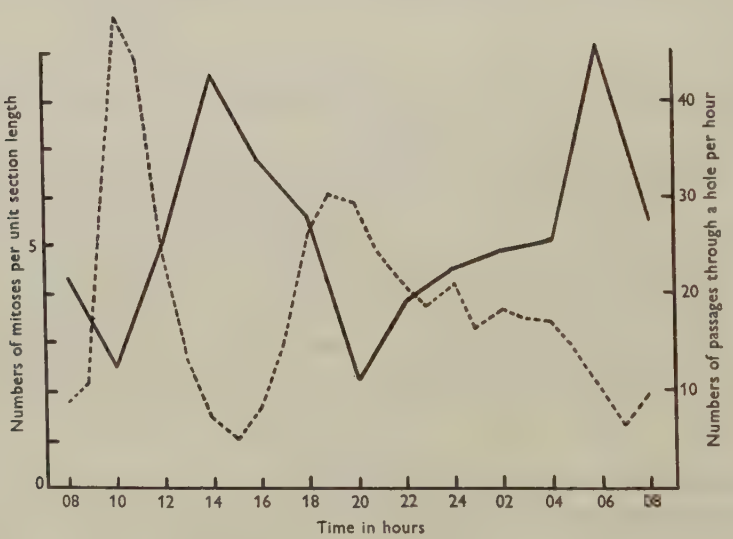

Fig. 4. The diurnal variations in the mitotic activity of the ear epidermis (solid line) as compared with the diurnal variations in the spontaneous bodily activity (broken line) of mature Strong's *CBA* males.

Middle age

The third experiment was performed with 17-month-old males, and the results are also shown in Table 12. They are illustrated graphically in Fig. 5.

The differences between this cycle of spontaneous activity and those of the younger animals are considerable. In the first place it can be seen that the middle-aged mice were not greatly disturbed when the food was put into their box at about 10.00 hr., so that the early morning rest period became almost continuous with the

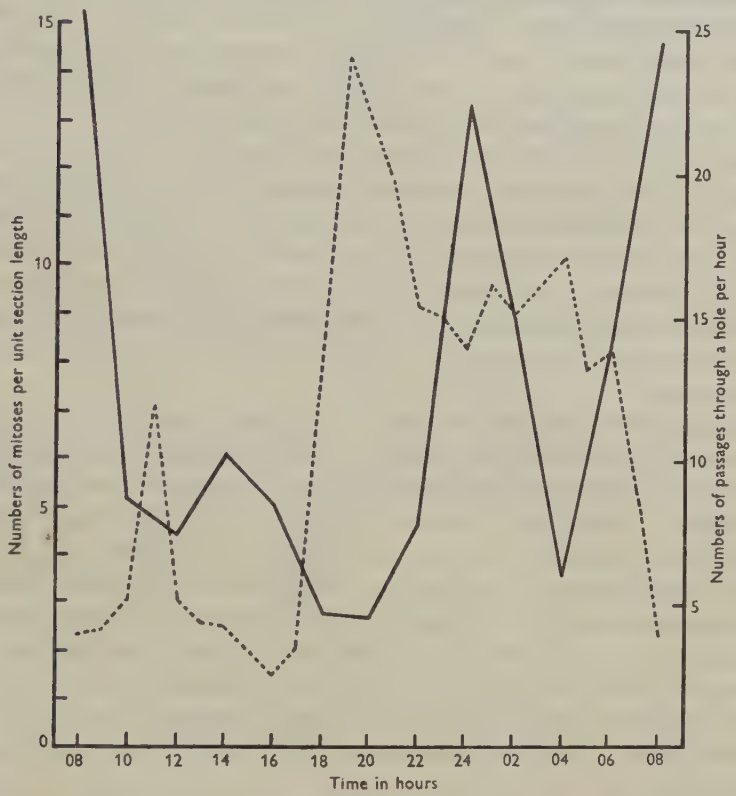

Fig. 5. The diurnal variations in the mitotic activity of the ear epidermis (solid line) as compared with the diurnal variations in the spontaneous bodily activity (broken line) of middle-aged Strong's *CBA* males.

afternoon rest period. The animals were closely watched at their feeding time, and it was found that they ate very little and quickly returned to rest. Thus their daily routine was a simple one with almost continuous rest during the 12 hr. period 06.00–18.00 hr., and almost continuous activity during the 12 hr. period 18.00–06.00 hr.

Another point of difference from the younger males was that at no time did these animals develop such a high rate of activity. The highest average number of passages through the hole in 1 hr. was only 23·9, as compared with 49·6 for the mature males and 46·4 for the immature males, while the lowest number of passages was 2·4, as compared with 5·0 and 11·1 respectively. This drop in the spontaneous activity of

the middle-aged mice is most clearly shown by the total of the average numbers of passages through the hole per day. This figure was only about 280, as compared with 460 for the mature males and 565 for the immature males.

In Fig. 5 the comparison is made between the bodily activity (Table 12) and the mitotic activity (Table 9) of middle-aged Strong's *CBA* males. Once again the usual inverse relationship is demonstrated. The beginning of the early morning rest period coincides with a rise in the mitosis rate, and the beginning of the evening activity coincides with a fall. In the night there is a slackening of bodily activity about midnight which is accompanied by a second rise in the mitosis rate, and an increase in activity about 04.00 hr. which is accompanied by a second fall. In addition, there are minor fluctuations in bodily and mitotic activity between 10.00 and 18.00 hr. which also show an inverse relationship.

However, these times of minor fluctuations by night and by day are particularly interesting since they provide an apparent contradiction. It can be seen that the rest period between 10.00 and 17.00 hr. is the longest and most clearly defined in the whole day, and yet it is accompanied by only relatively slight mitotic activity. By contrast, the reduction in bodily activity between 22.00 and 02.00 hr. is negligible, but the increase in the mitosis rate which accompanies it is great. This curious state of affairs has considerable theoretical importance, and it is dealt with in some detail in the next section.

With this apparent anomaly set aside, the general conclusions emerging from these results can be summarized as follows. In all age groups high bodily activity is associated with a low rate of mitosis, while rest or sleep is associated with a high rate of mitosis. Thus the differences observed in the timing of the diurnal mitosis cycles of immature, mature and middle-aged Strong's *CBA* males are related to, and apparently dependent on, differences in the timing of the diurnal cycles of spontaneous activity. Finally, it might appear to be significant that the immature animals which have the highest rate of bodily activity have also the lowest rate of mitotic activity; that the mature animals which have a lower rate of bodily activity have a higher rate of mitotic activity; and that the middle-aged animals which have the lowest rate of bodily activity have the highest rate of mitotic activity.

(5) *Mitosis during sleep in middle-aged mice*

As described above, the cycle of mitotic activity in the ear epidermis of middle-aged Strong's *CBA* males offers an apparent modification of the general rule. While the animals observed 12 continuous hours of almost uninterrupted rest, this did not result in 12 continuous hours of high mitotic activity. The mitosis rate rose to a very high level at the beginning of this rest period, but by 10.00 hr., when the mice were fed, it fell to a relatively low level. After the insignificant disturbance due to feeding, the mitosis rate rose only slightly at 14.00 hr., and then fell steadily until the end of the rest period. With the beginning of the evening period of wakefulness it fell still further.

In a previous publication (Bullough, 1949*a*) the tentative conclusion was reached

that the critical factor which allows the development of a high rate of mitosis is probably the high glycogen content of the tissue concerned, and further that such a high glycogen content is normally developed with the onset of sleep because of the deposition of blood sugar which takes place at that time. However, it may be surmised that the greatest deposition takes place only at the beginning of sleep while the blood sugar level is actually being lowered, so that the process is not a continuous one. Consequently it may well be that if sleep is unduly prolonged, so that the glycogen content of the tissue becomes depleted, the mitosis rate must fall. In a younger mouse the sleep period does not normally last for more than a few hours at a time, and it appears that sufficient energy is stored in the epidermis to maintain a high level of mitotic activity until the animal wakes. In a middle-aged mouse the sleep period is apparently too long for this to happen, and the slight disturbance at about 10.00 hr. is seemingly followed by only slight further deposition of sugar so that the mitosis rate shows only a slight recovery at 14.00 hr. After 14.00 hr. the fall in the mitosis rate is continuous until after the next burst of activity and of feeding. Then the relatively slight period of rest about midnight is accompanied by the development of a very high rate of mitotic activity.

If this theory is correct, it should be possible to cause an almost immediate rise in the mitosis rate of a sleeping middle-aged male by supplying extra carbohydrate by injection, since it could be expected that this carbohydrate would be taken up by the blood stream and deposited in the tissues. The following series of experiments was performed to test this. Sleeping Strong's *CBA* males, all middle-aged, were injected subcutaneously with starch solution using the technique developed by Bullough (1949*a*). This was done at 11.00 hr. when it was anticipated that the mitosis rate would be low, and each mouse received 20 mg. of starch dissolved in 0·4 c.c. of normal saline. Earclips were taken from these mice, and from saline-injected controls, at intervals from 08.00 to 20.00 hr., and it was observed that neither the injections nor the removal of the earclips caused any significant disturbance of the rest period. The results of four separate experiments are shown in Tables 13 and 14.

All the results were essentially similar. A single injection of 20 mg. of starch induced an immediate rise in the epidermal mitosis rate, so that between 1 and 3 hr. later a rate of cell division similar to that normally seen at about 08.00 hr. was

Table 13. *The effect of the injection of 20 mg. of starch at 11.00 hr. on the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten sleeping, middle-aged, Strong's CBA males.*

Time of day	Mice aged 15 months		Mice aged 16 months	
	Injected with saline	Injected with starch	Injected with saline	Injected with starch
08.00	15·2±0·58	14·7±0·79	14·1±0·32	12·7±0·35
10.00	4·5±0·31	3·9±0·30	5·4±0·23	4·9±0·30
12.00	3·7±0·13	8·3±0·39	3·9±0·21	8·1±0·33
14.00	4·6±0·20	12·4±0·44	4·7±0·25	11·1±0·45
16.00	3·5±0·13	4·3±0·32	2·4±0·18	5·1±0·33
18.00	2·7±0·16	2·1±0·22	1·1±0·24	2·7±0·18
20.00	3·2±0·28	2·9±0·23	2·0±0·16	1·2±0·26

Table 14. *The effect of the injection of 20 mg. of starch at 11.00 hr. on the average numbers of mitoses present in unit section lengths (1 cm.) of the ear epidermis in groups each of ten sleeping, middle-aged, Strong's CBA males*

Time of day	Mice aged 17 months		Mice aged 20 months	
	Injected with saline	Injected with starch	Injected with saline	Injected with starch
08.00	13·3 ± 0·37	13·6 ± 0·52	13·6 ± 0·41	13·4 ± 0·65
10.00	5·4 ± 0·35	8·2 ± 0·39	6·3 ± 0·39	7·6 ± 0·48
12.00	3·9 ± 0·13	10·2 ± 0·59	3·1 ± 0·19	13·8 ± 0·68
14.00	5·2 ± 0·18	12·0 ± 0·41	5·5 ± 0·21	9·6 ± 0·55
16.00	4·9 ± 0·23	4·1 ± 0·22	4·8 ± 0·23	5·4 ± 0·25
18.00	2·9 ± 0·09	4·6 ± 0·35	3·7 ± 0·15	4·5 ± 0·32
20.00	2·6 ± 0·15	5·5 ± 0·28	1·2 ± 0·16	1·0 ± 0·20

induced. This is in agreement with the theory that an exceptionally long sleep period results in a depletion of the carbohydrate content of the ear epidermis with a consequent drop in the rate of cell division.

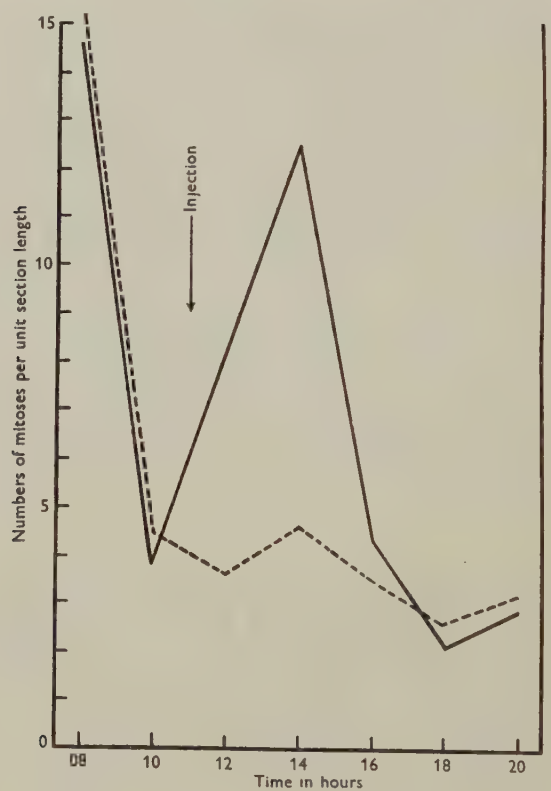

Fig. 6. The variations in the mitotic activity of the ear epidermis in resting, middle-aged, Strong's *CBA* males following the injection at 11.00 hr. of 20 mg. of starch in normal saline (solid line) and of normal saline alone (broken line).

(6) *Mitosis in other tissues*

Throughout this investigation it was found convenient to concentrate on conditions in the ear epidermis, but it is obviously of the greatest importance that an attempt should be made to discover whether the results obtained are also typical of other tissues. It was hoped that precise and detailed information on this point would become available from a study of the colchicine injected animals, but the difficulties encountered in the use of this drug have already been described. However, valuable, if limited, information was obtained from some of the tissues of the Kreyberg's white label mice which had given the most reliable results for the ear epidermis.

The first tissue to be examined from these mice was the epidermis of the antero-dorsal region of the back, the region above the scapulae. This was done in order to determine whether the conditions already found in the ear epidermis could be considered as typical of the epidermis as a whole. The mitoses were counted in unit lengths of 1 cm. of sections cut 7 μ thick, and the results, given in Table 15, are therefore directly comparable to those already obtained from the ear.

Table 15. *The average numbers of mitoses arrested by colchicine in 12 hr. in unit section lengths (1 cm.) of the antero-dorsal epidermis in groups each of ten Kreyberg's white label mice.*

Age of mice	Period 09.00–21.00 hr.	Period 21.00–09.00 hr.
1 month	48·5 ± 2·41	22·9 ± 1·13
5 months	39·8 ± 1·52	17·3 ± 1·08
16 months	44·5 ± 2·70	20·5 ± 1·21

In this table there is one result which is strikingly different from anything which has been described before, namely that the highest mitotic activity occurred in the 1-month-old mice. All the results obtained with the ear epidermis have shown that low mitotic activity is typical of the immature age group, and it follows that, in this particular at least, the ear epidermis cannot be taken as typical of the epidermis as a whole. However, the results for the middle-aged mice confirm those already obtained with the ear epidermis in showing an increase in mitotic activity over that recorded for the mature mice.

The second tissue examined was the stratified epithelium lining the oesophagus. Sections, 7 μ thick, were cut transversely in the region just anterior to the diaphragm, and the numbers of mitoses were counted in unit section lengths of 1 mm. The results are given in Table 16.

Table 16. *The average numbers of mitoses arrested by colchicine in 12 hr. in unit section lengths (1 mm.) of the stratified epithelium of the oesophagus in groups each of ten Kreyberg's white label males*

Age of mice	Period 09.00–21.00 hr.	Period 21.00–09.00 hr.
1 month	15·7 ± 0·53	12·6 ± 0·91
5 months	9·1 ± 0·49	6·7 ± 0·36
16 months	20·0 ± 0·98	16·4 ± 0·63

Again the immature mice gave a mitosis count which was considerably higher than that given by the mature mice, and again the middle-age period was characterized by a sharp rise in the mitosis rate.

The third tissue examined was that of the salivary gland. This was cut into sections 7 μ thick, and the mitoses were counted in unit section areas of 0·5 mm.^{2}. The results are shown in Table 17.

Table 17. *The average number of mitoses arrested by colchicine in 12 hr. in unit section areas (0·5 mm.^{2}) of the salivary gland in groups each of ten Kreyberg's white label males*

Age of mice	Period 09.00–21.00 hr.	Period 21.00–09.00 hr.
1 month	3·5 ± 0·22	1·6 ± 0·08
5 months	1·3 ± 0·06	0·7 ± 0·05
16 months	2·0 ± 0·07	1·1 ± 0·06

Once again the results are similar with a relatively high mitosis rate in the immature and middle-age groups.

The final tissue examined was the epithelium lining the tubules of the epididymis. This was chosen as a representative of the accessory sexual organs, and the counts were made on sections, cut 7 μ thick, of that region of the caput epididymis in which the epithelial cells have a particularly tall columnar form. For the present purpose the tissue was regarded as homogeneous, and the numbers of cell divisions were estimated in unit section areas of 0·5 mm.^{2}. They are recorded in Table 18.

Table 18. *The average numbers of mitoses arrested by colchicine in 12 hr. in unit section areas (0·5 mm.^{2}) of the epididymis in groups each of ten Kreyberg's white label males*

Age of mice	Period 09.00–21.00 hr.	Period 21.00–09.00 hr.
1 month	11·4 ± 0·41	5·9 ± 0·21
5 months	0·9 ± 0·11	0·7 ± 0·06
16 months	2·1 ± 0·15	1·6 ± 0·13

In spite of the fact that these figures do not really represent the numbers of mitoses occurring in a period of 12 hr., two conclusions emerge the validity of which can hardly be doubted. The first is that the ear epidermis is abnormal in developing fewer mitoses in the immature stage than in any other stage except the senile. The results for the other tissues indicate unanimously that mitotic activity is greater, and sometimes far greater, in the immature than in the mature male.

The second conclusion is that these results do not contradict the evidence of the ear epidermis that a rise in mitotic activity is typical of middle age.

IV. DISCUSSION

The main conclusion arising from the foregoing data is that, when judged from the point of view of mitotic activity, there are four distinct ages in the life of a male mouse. Of these it seems generally true to say that the first is characterized by a high rate of cell division, and, since the animals are actively growing at this time, this is what would be anticipated. The second age begins when the adult body size has been attained. Then there is an abrupt change to a lower rate of mitosis, which is maintained at a remarkably steady level until the age of about 12 or 13 months when a further change marks the onset of middle age. This third age is characterized by an increased mitosis rate, the precise degree of increase apparently varying from tissue to tissue. The final change occurs with the onset of senility, and, although fewer data are available concerning it, it is probably true, and certainly logical, to say that it is characterized by a mitosis depression which affects the whole body.

Apart from the evidence of the mitosis rate, the difficulty of defining these four ages is considerable. The state of the reproductive system is of no assistance, and no other internal criterion has been discovered except the size of the fat deposits. Externally it is often possible, and with practice usually possible, to distinguish the four ages by the size and general appearance of the animals. Thus immature mice have not yet reached their full stature, while middle-aged mice have exceeded it by the deposition of quantities of fat. Coincidentally, the immature animals are excitable and active, while the middle-aged animals are placid and quiet. The senile animals are feeble and shrunken with arched backs and poor fur, so that they are particularly easily distinguished.

This general vagueness of definition makes it difficult to suggest any obvious basis for the changes in the mitosis rate, and the curious fact that the change from age to age is apparently quite sudden adds to the difficulty. It is extraordinary how the transition from the immature to the mature plan of mitotic activity is accomplished in no more than a week or two, and the same phenomenon is evident in Kreyberg's mice during the transition from middle age to senility. The change from maturity to middle age is perhaps equally abrupt, but this is not yet certain.

Approximately coincident with these changes in the mitosis rate are the changes in spontaneous bodily activity. These can be regarded as furnishing a complete explanation for any alteration in the timing of the mitosis cycle, as, for instance, that between the immature and mature ages, and again that between the mature and middle ages in the Strong's *CBA* mice. However, in spite of the general inverse relationship which exists between bodily activity and mitotic activity, it seems unlikely that changes in spontaneous bodily activity alone can account for the observed alterations in the mitosis rate. The transition from mature to middle age is characterized by a great reduction in spontaneous bodily activity and by an increase in the mitosis rate, but, while the one may assist in the development of the other, it appears probable that it is not solely responsible for it. It is interesting to notice here that during pregnancy in the rat the spontaneous bodily activity is also greatly reduced (Wang, 1925). Again the reduction in muscular activity might be expected to favour the development of a high rate of mitosis, but obviously this reduction cannot be

held solely responsible for the raised mitosis rate. Further, it must be remembered that immature mice are very active and restless and yet have a high mitosis rate, while conversely senile mice spend almost the entire day lying at rest and yet only develop a low mitosis rate.

The conclusion seems inescapable that the age changes in the mitosis rate are due mainly to some factor other than that of exercise. In analysing this point it is obviously important to discover whether these changes, which are so abrupt in the ear epidermis, are equally abrupt and have the same timing in all other tissues. Such evidence as is available at the moment suggests that this may be so, and, if this is proved, then perhaps the critical factor or factors may lie not in the tissues themselves but in some discreet part of the body. In this case, a critical change in the composition of the blood might be suspected, and here it is of interest to recall the tendency for both the blood-sugar level and the renal threshold to rise with increasing age (see review by Cannon, 1942). From previous results on the effects of the blood-sugar level on mitotic activity (Bullough, 1949*a*), it would be expected that any such rise would be accompanied by an increase in the mitosis rate, and this might perhaps furnish some explanation for the condition in middle age. Of course, as already mentioned, the reduction in the spontaneous bodily activity during middle age may also assist in the development of excessive reserves of sugar, and the deposition of fat at this time might be taken as evidence that such an excess does, in fact, develop.

An interesting side issue which may be mentioned here is the effect on mitotic activity of the prolonged rests of the middle-aged animals. That rest and sleep are favourable to mitotic activity is now well known (Bullough, 1948*a*, *b*), but the present results show clearly that full stimulation is achieved only during the first few hours. Thereafter the mitosis rate falls unless more carbohydrate is added to the system for deposition into the tissues. This happens if the animal wakes, eats and sleeps again, or if carbohydrate is injected.

The fact that middle age is characterized by an increase in mitotic activity which is apparently general throughout the body is of particular interest. While male mice do not usually develop spontaneous tumours, it may be said of mice generally that the cancer age begins at about 12 months. If it should now transpire that an increase in the mitosis rate is normal during mammalian middle age, when spontaneous tumours are especially liable to develop, it may be a matter of considerable importance. Mottram (1944), and others working on experimental carcinogenesis, have distinguished between the blastogenic action of a carcinogen in producing cancer cells, and the developing action of non-carcinogenic factors which, by inducing hyperplasia, assist in the formation of a tumour. Thus Berenblum & Shubik (1947) have insisted that the initial action of a carcinogen is to induce a sudden and irreversible change whereby a few normal cells are converted into 'latent tumour cells' which then lie dormant. The development of these 'latent tumour cells' is an altogether different process which can be assisted by any treatment causing hyperplasia, and, in the absence of such treatment, many of these cells would never receive the stimulus to develop. Thus, while the development of a raised mitosis rate in middle age would not of itself be expected to cause, or even assist in, the formation of cancerous cells,

it might be expected to increase the chances of development of any latent cancer cells which were already present.

It would follow from this that if the high mitotic activity of middle age could be reduced, a reduction in the incidence of spontaneous tumours might also result. In this connexion it is now known that underfeeding has a powerful effect in reducing cancer incidence. The reviews of Tannenbaum (1947) and Boyland (1948) include evidence that a reduction in the diet of a mouse to two-thirds of what it would eat if it fed *ad lib.* markedly reduces the incidence of a variety of tumours, both spontaneous and induced, and also retards the time of appearance of those which do form. It has now been shown (Bullough, unpublished) that such starvation has the effect of causing an immediate and pronounced reduction in the mitosis rate of the ear epidermis of the male mouse, and thus it is evident that a restriction of diet acts in an opposite manner to that of a developing agent which induces hyperplasia.

It may therefore be suspected that any factor which restricts mitotic activity in middle age, and so induces what can be called hypoplasia, will also hinder the formation of tumours. At the moment the most potent restricting agents known are starvation and insulin, both of which act by lowering the blood-sugar level, and a similar effect can be induced by phloridzin, which acts by reducing the availability of whatever sugar is present in the body (Bullough, 1949*a*). While work on these lines is still in progress, preliminary results have already indicated that mice kept phloridzinized during middle age are considerably less liable to develop spontaneous tumours than are the controls. In view of these results it appears highly significant that, in the experiments reported by Tannenbaum concerning the effect of restrictions of diet on carcinogenesis, it is the carbohydrate fraction of the food which is the most important. A reduction in the protein fraction has no effect on tumour development, while a reduction in the fat fraction produces irregular results.

V. SUMMARY

1. A study has been made of the mitosis rate and of the diurnal cycles of male mice during each of the first 20 months of life. The mice used belonged to the Kreyberg's white label and the Strong's *CBA* strains. Most of the observations were made on the ear epidermis, but some attention was also given to other tissues.

2. It was discovered that, when judged from the point of view of mitotic activity, the life of a male mouse consists of four ages. During the immature age the animals are still growing and their mitosis rate is generally high, although the ear epidermis provides an exception to this rule. During the mature age which lasts from about the 3rd to the 12th month the mitosis rate is lowered. During the middle age which follows the mitosis rate increases, but in senility it is again reduced.

3. Coincident with these changes in the mitosis rate are changes in the spontaneous bodily activity. The mice are most active during immaturity and maturity. In middle age their activity is reduced by about half, and in senility they spend almost the whole time resting. Particularly in the Strong's *CBA* mice there are also changes in the timing of the diurnal cycle of spontaneous bodily activity, and these are immediately mirrored by changes in the timing of the diurnal cycle of mitotic

activity so that throughout life a general inverse relationship between bodily activity and mitotic activity is maintained.

4. In middle-aged Strong's *CBA* males the daily rest period extends almost without interruption from 06.00 to 18.00 hr. However, the most active cell division develops only at the beginning of this period, and it is evident that in prolonged sleep a lack of some vital factor develops. It is shown that subcutaneous injections of starch overcome this lack in sleeping mice and result almost immediately in the redevelopment of a high mitosis rate. Thus it would appear that sugar is the vital factor involved, and that the sugar content of the tissues is quickly used up during high mitotic activity.

5. These results are discussed particularly in relation to the problem of carcinogenesis.

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THE ACTION OF COLCHICINE IN ARRESTING EPIDERMAL MITOSIS

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(With Two Text-figures)

I. INTRODUCTION

In the foregoing paper (Bullough, 1949*b*) a doubt is expressed concerning the value of colchicine for collecting in the metaphase all those mitoses which normally occur during a 12 hr. period. It is probably true to say that most studies of animal mitosis made with the help of colchicine have been planned on two assumptions, namely that, within reasonable limits, the drug arrests every mitosis reaching the metaphase, and, equally important, that it neither increases nor decreases the number of resting cells entering mitosis. Following the pioneer work of Dustin (1934), Lits (1934) and others, these reasonable limits have been defined (see Allen, 1937) as a dose of 0·1 mg. given subcutaneously in water or normal saline and allowed to act for a period of $9\frac{1}{2}$ hr. With smaller doses only a partial stoppage of mitosis is achieved; with larger doses the resting cells are prevented from entering the prophase; and with longer periods the number of arrested mitoses does not increase because there is a complete stoppage of all cell division.

By means of the earclip technique (Bullough, 1948) it is now possible to test these various assumptions and conclusions, and the results of preliminary experiments to this end are recorded below. Each experiment involved a comparison between two groups of ten male mice of identical ages. The animals of one group were each injected subcutaneously with 0·1 mg. of colchicine dissolved in 0·25 c.c. of water, while those of the other each received 0·25 c.c. of water as a control. At the time of the injection, and at 2 hr. intervals thereafter, an earclip was removed from each animal by means of a conchotome. The last earclips were taken 12 hr. after the time of injection. The clips were fixed in alcoholic Bouin, cut into sections 7μ thick, and, after staining, the mitoses were counted in unit section lengths of 1 cm. The method of assessing the degrees of mitotic activity was the same as that described in the foregoing paper.

II. OBSERVATIONS

Each of the first two groups of mice consisted of ten 3-month-old Kreyberg white label males. They were injected at 08.00 hr., and they gave the results recorded below and in Fig. 1.

While the control animals show the variations typical of this part of the diurnal cycle with a peak of activity at about 14.00 hr. (Bullough, 1948), it is evident that the colchicine-injected animals developed most of their mitoses before that time.

A comparison between the two groups of figures shows that at 14.00 hr. the number of arrested mitoses in the colchicine-injected animals should not have been less than 7, whereas in fact it was observed to be only 4·5. It is therefore evident that some time between 12.00 and 14.00 hr. the colchicine had begun to depress the number of resting cells which were entering the prophase. However, a few prophases were still to be seen at 14.00 hr. so that in all probability cell division was not entirely inhibited until after this time. After 14.00 hr. no more prophases were seen in the colchicine-injected animals although they continued to be observed in the control animals.

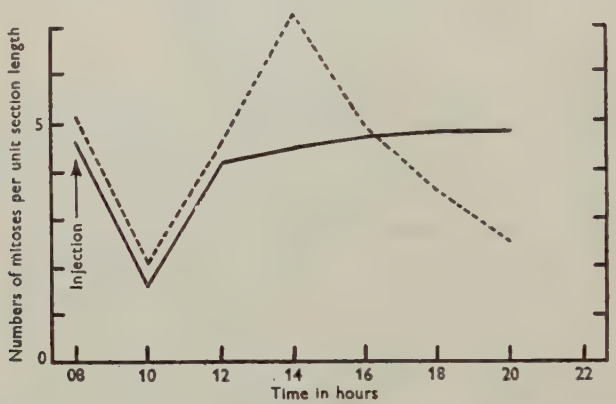

Fig. 1. The variations in the mitotic activity of the ear epidermis of mice injected at 08.00 hr. with colchicine dissolved in water (solid line) and with water alone (broken line).

Table 1. *The average numbers of mitoses present per unit length* (1 *cm.*) *of sections of the ear epidermis of adult male mice injected at* 08.00 hr.

Time of day	Mice injected with colchicine in water	Mice injected with water alone
08.00	4·6 ± 0·32	5·1 ± 0·25
10.00	1·7 ± 0·14	2·0 ± 0·39
12.00	4·2 ± 0·18	4·6 ± 0·23
14.00	4·5 ± 0·16	7·3 ± 0·30
16.00	4·7 ± 0·19	4·9 ± 0·19
18.00	4·8 ± 0·29	3·7 ± 0·23
20.00	4·8 ± 0·21	2·7 ± 0·21

It was also interesting to notice in the colchicine-injected mice that, while at 10.00 hr. only pro- and metaphases were to be found, at all subsequent hours both ana- and telophases were also present. Thus it was evident that many of the divisions succeeded in passing the metaphase, though whether any were able to reach the resting stage once more could not be determined.

An important practical consideration arises from these conclusions. It is obvious that if the drug is allowed to act for periods of up to $9\frac{1}{2}$ or 12 hr., the number of mitoses observed will depend not on the degree of mitotic activity normally occurring during these periods, but on the degree of mitotic activity occurring during the

first 5 hr. only. Thus, at least as regards the ear epidermis, the maximum time which an injection of 0·1 mg. of colchicine should be allowed to act in an adult mouse is 5 hr., or if it is allowed to act longer, then the result obtained should be regarded as referring only to the first 5 hr. period.

The practical importance of this is demonstrated in Table 2, which is based on the results of an experiment in all ways similar to the first except that the injections were given at 10.00 instead of 08.00 hr. These results are also expressed graphically in Fig. 2.

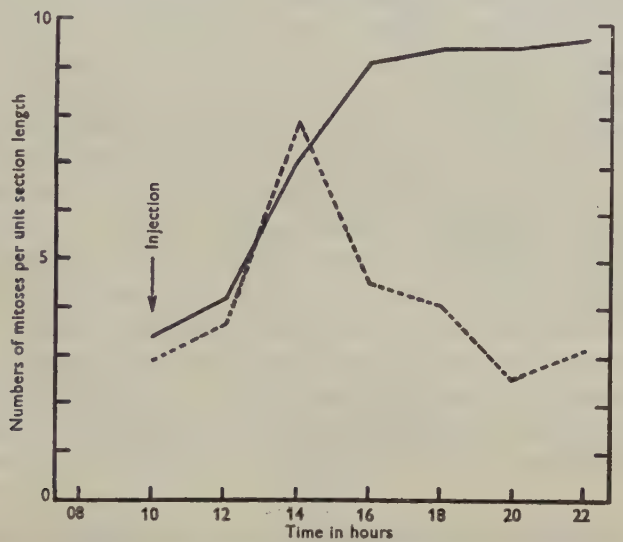

Fig. 2. The variations in the mitotic activity of the ear epidermis of mice injected at 10.00 hr. with colchicine dissolved in water (solid line) and with water alone (broken line).

Table 2. *The average numbers of mitoses present per unit length* (1 *cm.*) *of sections of the ear epidermis of adult male mice injected at* 10.00 *hr.*

Time of day	Mice injected with colchicine in water	Mice injected with water alone
10.00	3·4 ± 0·27	2·9 ± 0·21
12.00	4·2 ± 0·24	3·6 ± 0·37
14.00	7·1 ± 0·31	7·9 ± 0·25
16.00	9·3 ± 0·33	4·6 ± 0·19
18.00	9·5 ± 0·16	4·2 ± 0·19
20.00	9·5 ± 0·52	2·7 ± 0·20
22.00	9·7 ± 0·55	3·2 ± 0·15

Once again the colchicine only arrested mitoses in or after the metaphase during a period of about 5 hr. After 6 hr. no more prophases were discovered, and it is probable that no more resting cells were entering into division. However, since in this experiment the first 5 hr. coincided exactly with the rise in the mitosis rate which is normally associated with the early afternoon sleep period, twice as many divisions were arrested as in the first experiment.

A comparison between the control mice in the two experiments shows that the

number of divisions normally occurring in the two 12 hr. periods 08.00–20.00 hr. and 10.00–22.00 hr. was about the same. In both cases the average figures add up to about 30. Thus it was merely the 2 hr. difference in the time of injection which was responsible for the apparent doubling of the mitosis rate in the colchicine-injected animals of the second experiment. This result can only throw doubt on those published accounts of mitotic activity in which the period of colchicine action has been longer than 5 hr., and in which no account has been taken of the diurnal cycle.

During this work it was accidentally observed that the administration of 0·1 mg. of colchicine to an adult mouse induces a severe depression of the blood-sugar level. This fact does not appear to have been observed before, and the reason for it is unknown. Following the discovery of the direct connexion existing between the availability of sugar and the rate of mitosis (Bullough, 1949*a*), it was suspected that the depression of mitotic activity after the first 5 hr. period might be connected with this depression in the blood-sugar level. Consequently a series of observations was made on the effect of colchicine on blood sugar.

The same technique as before was adopted, the experimental mice being injected at 08.00 hr. with 0·1 mg. of colchicine in 0·25 c.c. of water, and the control mice with 0·25 c.c. of water alone. The technique by which the samples of blood were taken and their sugar content estimated is described by Bullough (1949*a*), and once again it is a pleasure to mention the assistance received from Dr A. Jordan who arranged for the blood-sugar estimations to be made in the laboratories of the Sheffield Royal Infirmary. Clearly the drop in the blood-sugar concentration, very

Table 3. *Variations in the blood-sugar concentration following an injection of colchicine at* 08.00 *hr.*

Time of day	Mice injected with colchicine in water		Mice injected with water alone	
	No. of observations	Blood-sugar level in mg. per 100 c.c.	No. of observations	Blood-sugar level in mg. per 100 c.c.
08.00	10	148·3 ± 5·12	10	151·1 ± 5·07
14.00	8	168·0 ± 4·71	10	147·9 ± 4·13
20.00	20	115·9 ± 3·93	10	182·8 ± 4·76

apparent after 12 hr., had not developed after only 6 hr., and indeed there are signs that an actual rise had occurred at that time. It follows that the depression of mitotic activity, which begins about 5 hr. after the injection of colchicine, may well be independent of changes in the blood-sugar concentration.

III. CONCLUSIONS

1. It is evident from the above experiments that for a period of about 5 hr. after the injection of 0·1 mg. of colchicine into an adult mouse there is no discernible change from the normal in the epidermal mitosis rate, and that during this period all, or most, of the epidermal mitoses are arrested in some stage after the prophase.

After this 5 hr. period the colchicine inhibits mitotic activity by preventing any more resting cells from entering the prophase.

2. During the first 5 hr. the arresting action of the colchicine is not felt until a division reaches the metaphase. However, considerable numbers of divisions succeed in passing this stage to reach the ana- and telophases, and it is possible that some of these divisions are completed.

3. After the first 5 hr. the mitosis inhibiting effect of colchicine is associated with severe nervous depression which ultimately may end in death. It is possible that both effects may be dependent on the formation of a toxic oxidation product, oxydicolchicine (Goodman & Gilman, 1947). It is also noted that colchicine depresses the blood-sugar concentration, but that this depression does not become apparent until after 6 hr. have passed.

4. The practical importance of these results is considerable. It is evident, at least in the case of the ear epidermis, that for a study of normal mitotic activity colchicine must not be allowed to act for more than about 5 hr. The period of $9\frac{1}{2}$ hr. suggested by Allen (1937) is almost twice too long, and, due to the complication of the diurnal cycle, the use of such a long period may yield the most anomalous results.

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THE EFFECT OF CALCIUM ON THE AXOPLASM OF GIANT NERVE FIBRES

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(With Plate 8 and One Text-figure)

As was first described by Bear, Schmitt & Young (1937), a jelly-like and apparently solid cylinder of axoplasm is obtained when the contents of a fresh giant nerve fibre of the squid are extruded. There are several observations which indicate that the axoplasm is also normally in a gelatinized state and behaves like a solid.

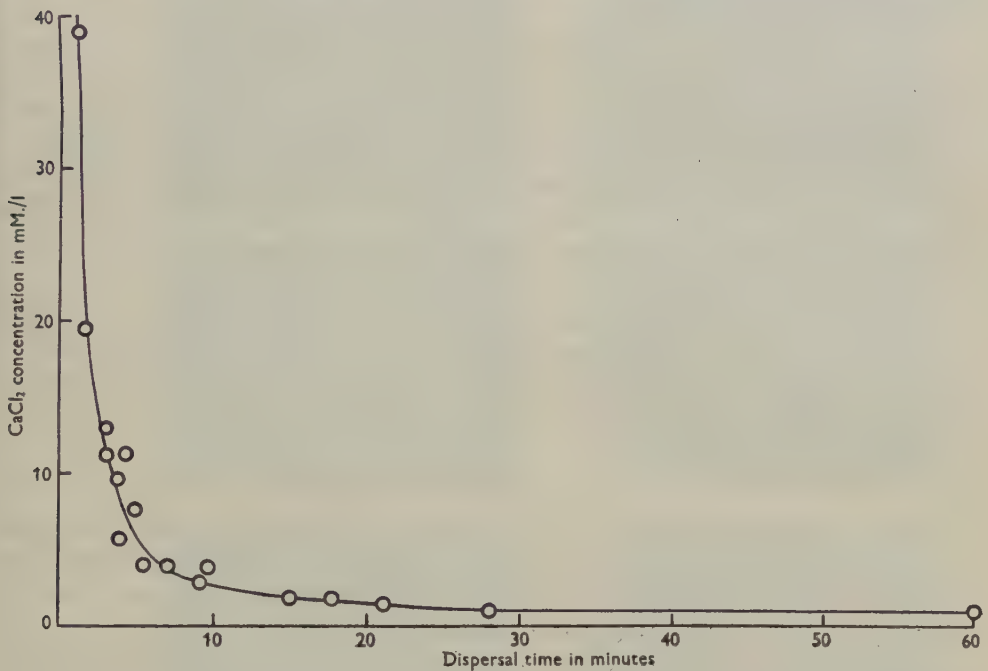
Thus, when a fresh 600μ axon is cannulated (Hodgkin & Huxley, 1945) and a 100μ capillary tube, filled with fluid and connected to a pressure reservoir, is thrust through the cannula several millimetres deep into the axon, no flow can be produced even with a pressure of 200 mm. Hg. When the capillary is withdrawn from the axon, a clear channel is frequently left behind which acts as a 'guide' for microelectrodes subsequently inserted into the axon. When chlorided silver electrodes are inserted, small particles of the electrode coating sometimes break off, and on withdrawing the wire these particles are seen to sink to the bottom of the cavity which the electrode has fashioned inside the axon.

While observations of this kind were usually made on fresh fibres, there were variations between different axons and, in particular, it was clear that prolonged exposure to sea water tended to reduce the firm consistency of the nerve fibres. Furthermore, injured regions of the axons quite commonly not only lost their excitability but became liquefied, and their contents flowed out when the axon was cut.

Bear *et al.* (1937) noted that the axoplasm extruded into sea water did not remain in its initial solid state but within a short time dispersed almost completely. We have confirmed this observation, but found that no such disintegration takes place when the contents of a fresh axon are extruded into a *Ca-free* solution of NaCl or KCl (0·58 g. mol. wt. per 1000 g. H_2O). Further experiments confirmed that the addition of a few millimols of $CaCl_2$ to a NaCl solution or to a Ca-free artificial sea water (Pantin, 1946, p. 63) caused the immersed axoplasm to disperse promptly. The effect is illustrated on two samples in Pl. 8. Pl. 8, *a* was obtained after the samples had been in a 30 c.c. bath of a 0·58M-NaCl solution for about 20 min. during which time their appearance did not change. Between exposures *a* and *b*, a small quantity of $CaCl_2$ (1 c.c. of a 0·39M solution) was added to the bath, and within a few minutes the outlines of the axoplasm became blurred, and it disintegrated (Pl. 8, *c–e*).

To obtain a more quantitative estimate of the calcium effect, the following procedure was used. A giant axon was divided into portions several mm. long, and

their axoplasm was extruded into a Petri dish filled with a 0·58M-NaCl solution. The individual pieces of axoplasm were then transferred by a pipette into a series of watch glasses containing mixtures of 0·58M-NaCl and 0·39M-$CaCl_2$ solutions. The watch glasses were shaken gently at half-minute intervals, and the time taken for a 'complete dispersal' of the axoplasm was recorded. The dispersal took place gradually, but the moment when no coherent mass of axoplasm remained visible could be determined with reasonable accuracy, and the method gave reproducible results. Control pieces were kept in the NaCl solution; they remained visible for more than an hour after the other samples had dispersed. Results of five experiments, at approximately 20° C. are plotted in Text-fig. 1. The dispersal time increased as



Text-fig. 1. The relation between the calcium concentration and the time required for complete dispersal of the axoplasm.

the calcium concentration was lowered. The extreme range observed was 0·5 min. in 390 mM. $CaCl_2$ and 100 min. in 0·71 mM. $CaCl_2$, these time values being accurate to about 20%. It is clear that one-tenth of the calcium concentration of sea water is sufficient to ensure a complete dispersal of the axoplasm.

With very high concentrations of CaCl (390 mM.) a twofold effect was seen: the axoplasm disintegrated completely within half a minute if the bath was stirred; but in the absence of stirring, the process stopped in its initial stage, and an apparently irreversible precipitate was formed which could not be dispersed by subsequent stirring or exposure to lower calcium concentrations.

The action of calcium appeared to be specific, for none of the other constituents of sea water had a similarly striking effect. When fairly high concentrations of

$MgCl_2$, up to 370 mM., were used the axoplasm gradually lost its sharp boundary, but no complete dispersal was observed even after a period of immersion thirty times longer than required with an equivalent concentration of $CaCl_2$. In an experiment in which eight samples of axoplasm were immersed in solutions isotonic with sea water and containing apart from NaCl 4, 13, 38 and 76 mM. of $CaCl_2$, or $MgCl_2$, respectively, all the 'calcium samples' had disintegrated within 5 min., while all the 'magnesium samples' were still visible in a coherent form after 36 min. In another experiment, the effects of $MgCl_2$, $CaCl_2$, $SrCl_2$ and $BaCl_2$ were compared, the concentration of each being approximately 4 mM. in a 0·58 M-NaCl solution. The calcium sample completely dispersed in 7 min., while the other samples remained visible after 35 min.

The dispersing action here described is similar to the liquefying effect of calcium on the protoplasm of Stentor (Heilbrunn, 1923), but it apparently contrasts with the coagulation which calcium is known to produce in other colloidal materials (Höber, 1920; Heilbrunn, 1943). It is possible that the disintegration of the axoplasm is brought about by a precipitating reaction between calcium and some substance which composes the frame of the colloidal structure. A chemical analysis of the fine residue which is formed in the course of the dispersal may provide further information on the nature of this effect.

The experiments throw an interesting sidelight on the concentration of calcium ions in the normal axoplasm. There is evidence that the axoplasm is normally in a gelatinized state, and this seems incompatible with a concentration of ionized calcium of more than about 500 μmol./l.

SUMMARY

1. Observations are described which indicate that the axoplasm of the giant nerve fibre of the squid is normally in a gelatinized state and behaves like a solid.

2. Extruded axoplasm disperses rapidly in sea water, but not in calcium-free solutions of 0·58 M-sodium chloride or potassium chloride.

3. The addition of calcium chloride in concentrations of 1 mM. or more causes the axoplasm to disperse, the time for complete disintegration decreasing as the calcium concentration is raised. No comparable effect is produced by the other constituents of sea water.

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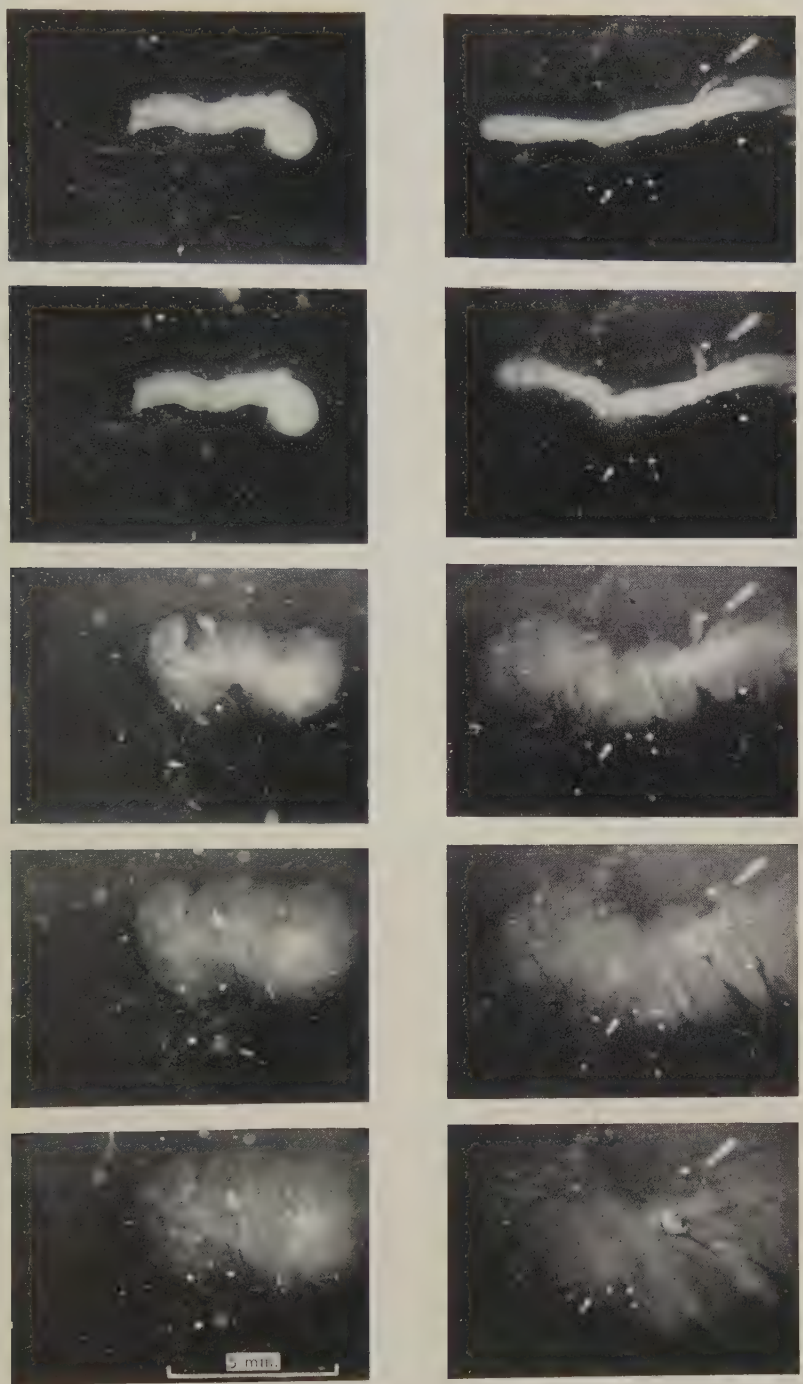
EXPLANATION OF PLATE 8

The dispersing effect of calcium on the axoplasm of squid nerve. Photographs taken with dark-ground illumination. For further description see text.



HODGKIN AND KATZ—THE EFFECT OF CALCIUM ON THE AXOPLASM OF GIANT NERVE FIBRES

STUDIES ON ANIMAL CAROTENOIDS

II. CAROTENOIDS IN THE REPRODUCTIVE CYCLE OF THE BROWN TROUT

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(*Received* 14 *March* 1949)

(With Three Text-figures)

In the first paper of this series (Steven, 1948) it was shown that the tissues of the trout (*Salmo trutta* Linn.) normally contain β-carotene, lutein and astacene. Lutein and astacene occur as esters in the red and yellow chromatophores of the skin, and may also be present in the muscles as free hydroxy-carotenoids. Carotene is present in the liver, which also contains xanthophylls but no astacene. The ripening oocytes, however, contain all three types of carotenoid. Red and yellow chromatophores first appear during the larval period of development before the young fish has commenced to feed independently, and appear to obtain their pigment from the carotenoids laid down in the yolk. This paper records the chemical changes and distribution of these substances from the maturation of the oocytes to the stage of metamorphosis of the larva.

Adult trout were obtained alive from a reservoir near Edinburgh. Some ova were obtained from ripe fish in the laboratory, and fertilized with sperm from males of the same batch. Larger numbers were supplied by the Howietoun and Northern Fisheries, Stirling, as 'eyed' ova, and were used for most of the experiments on the larval period of development. Ova and larvae were reared in batches of about 100 each in glass aquarium jars, which were immersed nearly to the rim in a larger tank through which passed a rapid flow of water taken direct from the mains supply. This arrangement gave satisfactory temperature stabilization for the period of the experiments, from January to the end of March. Temperature variation was less than 1° C. for periods of more than a week, and remained within the range 4–6° C. for the whole period.

Carotenoids were estimated with a photoelectric colorimeter by the method described in detail by Steven (1948).

THE MOBILIZATION OF CAROTENOIDS IN SPAWNING FEMALES

The ovaries of mature female brown trout vary greatly in size at different seasons of the year. In Britain the species spawns in late autumn, and during the winter months following the ovaries remain minute. In April and May they contribute about 1 % to the total body weight, and the oocytes which will ripen in the following autumn are each about 1 mm. in diameter and bright orange in colour. By October the combined ovaries may constitute up to 10 % of the body weight; the individual ova at the time of spawning are 5–6 mm. in diameter and weigh about 0·1 g. The changes found in their carotenoid content during the ripening period are summarized

in Table 1. The concentration of ovarian pigments, expressed as μg./g. of fresh tissue, was found to remain more or less constant during the period of maturation. The total amount, however, increases greatly as the oocytes enlarge, and can be regarded as an increase in the carotenoid content of each oocyte in proportion to its increase in size. The ratios of β-carotene, lutein and astacene also remained approximately constant during the maturation period.

Table 1

A. *The distribution of carotenoids in unripe and spawning female trout*

	Fish no.	Wt. of fish (g.)	Wt. of ovaries (g.)	Ovaries wt. % / Body wt.	Total carotenoids (μg./g.), estimated as lutein		
					Ova	Muscles	Skin and fins
Unripe females (May)	1	204	1·56	0·76	152	32	135
	2	253	1·90	0·68	114	17	166
	3	188	1·31	0·70	125	30	140
Spawning females	4	227	44	17·6	105	Trace?	172
	5	316	76	24	120	0	129
	6	208	48	23	102	0	155

B. *The detailed composition of ovary carotenoids*

		Carotenoids (μg./g. fresh tissue)			
		β-Carotene	Lutein	Astacene	Total carotenoids (estimated as lutein)
Small oocytes (May)	Fish no. 1	4	22	140	152
	Pooled sample from 8 fish	2·5	20	117	108
Near-ripe oocytes (Sept.)	Pooled sample from 3 fish	2·5	13	160	112
Ripe ova (Nov.)	Fish no. 5	3·5	15	126	129
	Fish no. 6	2·0	24	137	155
	Pooled sample of 5 fish	3·5	18	124	102

It was shown previously (Steven, 1948) that the muscles of trout may contain up to about 30 μg./g. of astacene and 3·5 μg./g. of lutein. Those of ripe female fish examined shortly before or after spawning, however, yielded no carotenoids whatever. It seems clear that the free astacene and lutein of the muscles are transferred to the oocytes during the last few weeks before spawning, at the time when the latter are growing most rapidly. The astacene and lutein esters in the chromatophores of the skin, on the other hand, are not depleted, since the skins of ripe female fish yielded the same amounts of these pigments as did the skins of males and non-spawning females. The carotenoid content of the skin appears to be maintained at a constant level throughout the year in both sexes. If it is assumed that the muscles constitute about 60 % of the total body weight, those of a well-pigmented fish may contribute rather more than half the total lutein and astacene found in the ripe ova. Since the chromatophores are unaffected, and the fish does not possess any other considerable reserve of these pigments, the rest is presumably obtained direct from the food eaten during the months when the oocytes are increasing in size.

THE DISTRIBUTION OF CAROTENOIDS DURING THE LARVAL PERIOD

The distribution of carotenoids as between the yolk and the body of the embryo was measured at various stages from the time of hatching until metamorphosis. The procedure adopted with slight modifications for separating the yolks from the embryos was that described by Gray (1926). Batches of about fifty larvae were narcotized with 2 % urethane, their lengths measured individually to the nearest 0·5 mm., adherent water removed by drying lightly between sheets of filter-paper, and the group weighed as a whole. The yolk sacs were removed from the embryos, which were then washed with 0·7 % NaCl, again dried lightly with filter-paper and reweighed. The washing detached any droplets of pigmented yolk fat adhering to the embryos. These droplets were collected from the surface of the saline solution and returned to the yolk fraction. The embryos and yolks were then ground separately with anhydrous sodium sulphate, extracted exhaustively with petroleum ether containing about 2% of methyl alcohol, and the carotenoids estimated by the usual method. In most experiments only the total carotenoid content of each fraction was measured, the result being expressed in terms of a standard solution of lutein, which was prepared from a crystalline sample supplied by Prof. L. Zechmeister. In some cases, however, the astacene and lutein were separated and estimated individually.

In order to detect any possible loss of pigment during the separation of embryos from their yolk sacs, and to obtain information on the carotenoid content of the whole larva at all stages of development, a second batch of about fifty larvae was taken from the same tank at the time of each experiment, weighed, dried and the total pigments extracted and estimated.

The carotene content of the yolk is much less than the lutein and astacene, and larger batches of up to 200 larvae were required to obtain reasonably accurate estimates of it.

The information obtained from this series of experiments is expressed in Table 2 and Fig. 1, which illustrate the following points:

(1) Lutein and astacene are transferred progressively from the yolk to the embryo during the larval period without apparent loss.

(2) The transfer of carotenoids is relatively delayed compared with the general development of the embryo or the rate of utilization of the yolk. At the time of hatching the embryo constitutes about 19 % of the total weight of the larva, but contains only about 7 % of the carotenoids. During the larval period, however, the rate of transfer of pigment increases steadily relative to the weight increase of the embryo, and at metamorphosis all the pigment is in the body of the embryo.

(3) Lutein and astacene are apparently transferred in constant ratio throughout the larval period.

(4) Although carotene constitutes about 2 % of the initial yolk carotenoids, none was detected in the embryo at any stage.

The rate of transfer of lutein and astacene corresponds closely with the visible development of xanthophores and erythrophores in the skin of the embryo. These types of chromatophore are not usually apparent at the time of hatching, although lipoid containing cells of typical embryonic chromatophore pattern can be demonstrated in the fins by staining with Sudan IV or Sudan Black by the method described by Baker (1945). The first pale yellow xanthophores can usually be seen a few days after hatching on the tail, dorsal and future adipose fins. They increase rapidly in

Table 2

A. *The transference of carotenoids from yolk to embryo during development at* 4–6° C.

Stage	% carotenoids in embryo	Embryo wt. / Larva wt. %	Total larval carotenoids (μg./larva)	Length of posterior end of embryo (mm.) (dorsal fin to tail)	Total length of embryo (mm.)
1. Freshly fertilized ova	0	0	9·8	0	0
2. Hatching	6·9	19	10·2	7·5	15·0
3.	9	33	8·0	8·5	17·5
4.	25	44	10·4	9·0	19·5
5.	48	68	7·3	11·5	23·0
6.	64	81	10·8	13·0	24·0
7.	80	89	12·0	14·0	24·5
8. Metamorphosis	100	100	10·0	15·0	25·0

B. *The detailed composition of embryo and yolk carotenoids* (μg./*larva*)

Stage	Yolk			Embryo		
	β-Carotene	Lutein	Astacene	β-Carotene	Lutein	Astacene
2	0·2	1·5	8·1	0	0·2	1·0
5	0·05 ?	1·0	4·6	0	1·3	4·4
8	0	0	0	0	1·9	11·2

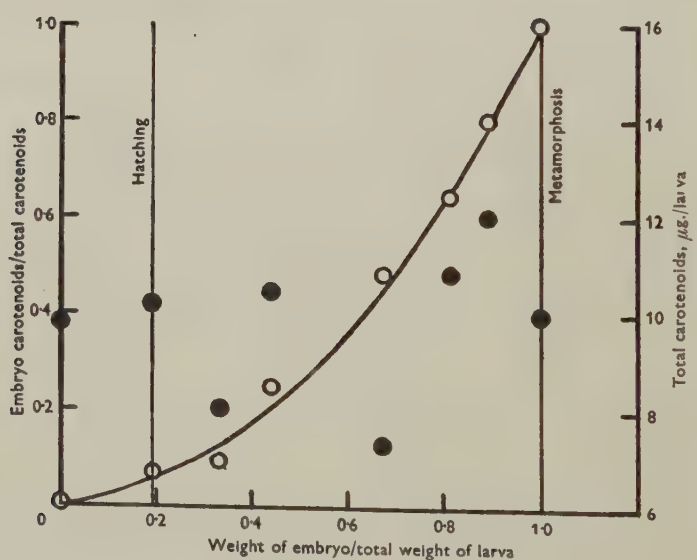

Fig. 1. To show the relation between the rate of transference of carotenoids from the yolk to the embryo and the general growth rate of the embryo (open circles). (The filled circles represent the values obtained for total larval carotenoids (yolks + embryos) at different stages from hatching to metamorphosis.)

number and intensity of pigmentation; and during the second week of larval life it is possible to distinguish the erythrophores, which appear first orange and then red. By metamorphosis both types are numerous, and distributed in accordance with the characteristic colour pattern of adult trout, erythrophores being confined principally to the red areas of the adipose and tail fins, while xanthophores are generally distributed over the surface of the body. Red spots along the lateral line are not, however, apparent during larval life.

The lutein and astacene of the yolk are in the form of free hydroxy-carotenoids. In the embryo, however, they were found to be esterified. In order to determine the distribution of these esters in the embryonic body, a number of larvae close to

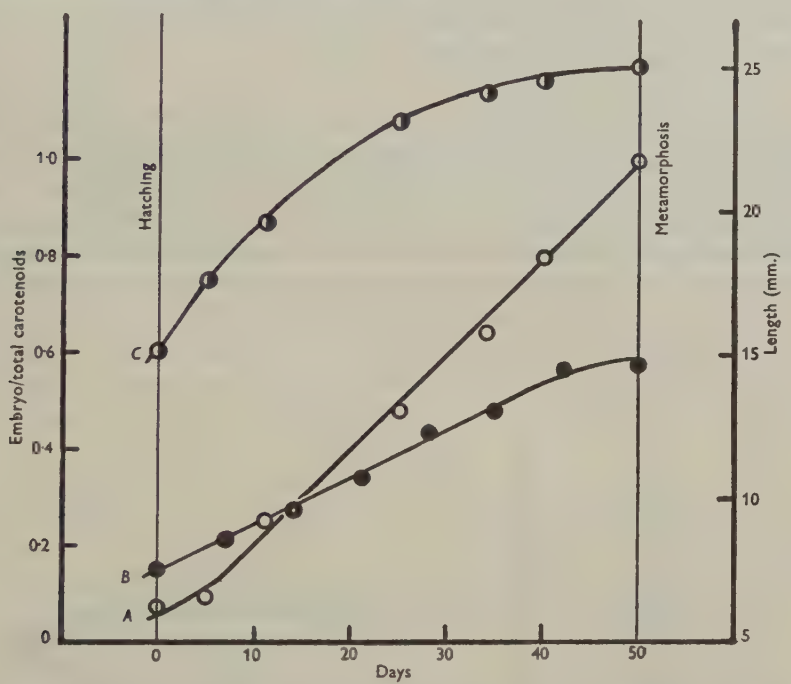

Fig. 2. To compare the rate of transference of carotenoids from yolk to embryo (A) with the growth in length of the posterior end of the embryo (B) and the growth in length of the whole embryo (C).

metamorphosis were skinned and the carotenoids of the whole skin, together with the fins, estimated separately from those of the rest of the carcass. The skin and fins were found to contain 64 % of the total carotenoids of the embryo. This represents the fraction of pigment laid down in the xanthophores and erythrophores by the end of the larval period. It was not possible to analyse further the distribution of the remaining fraction of pigment among the tissues of the rest of the carcass, but this, too, was in the esterified form. Further experiments will be required to establish the actual site where esterification takes place, which may prove to be in the yolk before the pigments are transferred, or possibly in the liver or in a variety of tissues of the embryo.

Although the transfer of lutein and astacene from the yolk is at first delayed relative to the general development of the embryo as measured by increase in weight, the process bears a general resemblance to the growth in length of the posterior end of the body, measured from the leading edge of the dorsal fin to the tip of the tail. Both are nearly linear with respect to time during the larval period, whereas the overall growth rate of the embryo, measured either as growth in length or weight, shows progressive deceleration (Fig. 2). As is well known, development of the head region is relatively precocious in Vertebrates. Most of the carotenoid containing chromatophores, however, develop in the skin of the posterior end of the body, particularly on the tail and adipose fins, and the similarity between the rate of transfer of pigment from the yolk and the growth of the posterior end may simply express the fact that the speed of transfer of pigments is related to the development of the cells which are to receive them.

THE EFFECT OF REMOVAL OF CAROTENOIDS FROM THE YOLK

Newly hatched larvae were sucked into lengths of glass tubing wide enough to hold them comfortably but prevent them from turning, and kept in water with their head ends directed downward. Being more buoyant than the aqueous part of the yolk, the oil globules containing the carotenoid rise in the course of a few minutes to the posterior end of the yolk sac, which projects from the body of the embryo. In many larvae almost

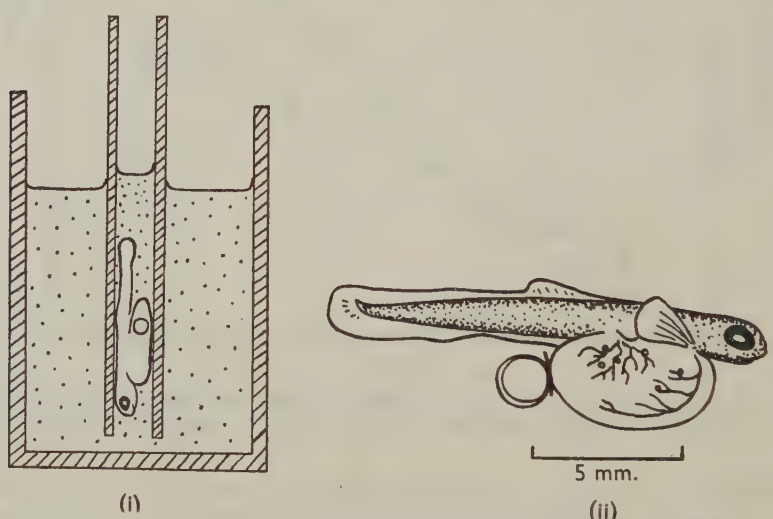

Fig. 3. Operation for removal of fat droplets containing carotenoids from the yolk sacs of trout larvae. (i) Alevin held vertically in tube. Fat globule rising to posterior end of yolk sac. (ii) Alevin with main fat globule of yolk ligatured. Small droplets of fat adhering to vitelline vessels.

all the pigment is contained in a single large globule. The larvae were narcotized with 2 % urethane while still in the head-down position. They were then removed from the tubes, and the oil globule ligatured with fine silk thread and cut away from the rest of the yolk (Fig. 3). The ligature was removed after a few days. By this operation almost the whole of the lipoid fraction and carotenoids were removed with a minimum amount of other yolk substances. In a second series of larvae a similar fraction of the yolk was ligatured

and removed in the same way, omitting the preliminary period in the vertical position, so that the pigment and oil globules were undisturbed and remained within the reduced yolk sac.

It proved possible by this operation to remove about 90 % of the lipoid and carotenoid of the yolk. Small fat droplets, some of them containing pigment, did not rise to the posterior end of the yolk sac, but remained attached to the walls of the vitelline blood vessels, from which they could not be detached by more severe procedures, such as centrifuging at low speeds, without killing the larvae. The amount of lipoid and carotenoid removed from the second series of larvae was less than 5 % of the total yolk content.

Post-operative mortality of both experimental and control series of larvae was about the same, and due mainly to rupture of the yolk sac or invasion by water at the site of the wound. The survivors of both series developed at the same apparent rate, though as noticed by Gray (1928) they were slightly smaller than unoperated larvae kept at the same temperature. Xanthophores and erythrophores developed in the control series as in normal larvae. Those from which the lipoid and carotenoid had been removed, however, developed very few chromatophores, and those which did appear contained little pigment. Pale yellow chromatophores were generally distributed over the fins and body surface before metamorphosis, but could not be differentiated as xanthophores and erythrophores. The experiment was not continued beyond this stage. The type of diets on which alevins are usually reared, such as chopped liver, yolk of egg or live Entomostraca, all contain considerable amounts of carotenoids, and an attempt to wean them on a low carotenoid diet of egg albumen, cod muscle and oatmeal proved unsuccessful.*

DISCUSSION

Hartmann, Medem, Kuhn & Bielig (1947) have recently analysed the chemical constituents of the ova of the rainbow trout, *S. irideus* Gibb. They found them to contain the same three carotenoids as the brown trout used in my investigations, but in considerably smaller amounts and in different relative proportions. 13,500 ova weighing 600 g. yielded only 350 μg. of pigment, estimated as lutein; and after separation the amounts of lutein, astacene and β-carotene were estimated as 43·4, 7·2 and 12·6 μg./100 g. of fresh ova respectively. Although the method of separation used by them involved several stages of purification by chromatographic adsorption and subsequent elution of the pigment fractions, a process which usually involves considerable loss of carotenoids by oxidation, the discrepancy between their values and those found by myself (Steven, 1948) for the brown trout appear to be too great to be accounted for solely by differences of procedure. MacWalter & Drummond (1933) found that the ova of rainbow trout contain less carotenoid and more vitamin A than those of brown trout, and it seems reasonable to conclude that the two species do in fact differ in the amounts and proportions of carotenoids laid

* Since this paper was sent to press a number of larvae, from which the carotenoids had been removed, have been reared successfully on live Enchytraeus. At the time of writing (June 1949) they completely lack xanthophores and erythrophores and are exceptionally pale in colour. In all other respects (growth rate, behaviour, etc.) they appear to be normal.

down in the ova, though not in their nature. The difference is greatest in the case of astacene, which is the most abundant carotenoid in the ova of *S. trutta*, but the least in *S. irideus*.

In this connexion it is important not to overlook the possibility that qualitative and quantitative differences found in different investigations may be due to differences in the diet of spawning female fish. Ova from different females vary considerably in carotenoid content, and it might be possible, as in the case of the hen, to produce ova with little or no pigment by supplying the fish a carotenoid-free diet for several months before spawning. There appears to be no experimental evidence on this point in the case of fish, although other experiments of mine (Steven, 1948) indicate that trout cannot synthesize any carotenoid and must therefore depend upon their food for their whole supply. It is worth noting, however, that the conditions under which trout are commonly reared in hatcheries, where they often receive a diet of a single foodstuff, such as horseflesh, may provide just the type of conditions required for producing ova with abnormally low carotenoid contents.

The most important feature of the investigations of Hartmann and his colleagues, however, is their discovery that in the rainbow trout astacene acts as a fertilization hormone (*Befruchtungsstoffe*) in a manner similiar to the action of echinochrome A in the sea urchin, *Arbacia pustulosa*. Concentrations of colloidal solutions of $1:10^5$ were found to be sufficient to activate spermatozoa and to sustain positive chemotaxis. Lutein and β-carotene had no such effect. No experiments have been described to test whether any of the carotenoids has a similar role in *Salmo trutta*. My findings emphasize the importance of the pigments in another function during the reproductive cycle. The amounts of lutein and astacene in the ova of brown trout are much greater than one would expect to be necessary if they acted solely as fertilization hormones. Moreover, all the available reserves of the parent female, principally the free hydroxy-carotenoids of the muscles, are mobilized and transferred to the ripening oocytes. The lutein and astacene esters of the chromatophores in the skin, however, are not depleted, and the external colour pattern of the fish is therefore unaffected. The pigments of the ova are not destroyed during development, but are transferred without loss into the body of the embryo, where a large proportion can be recovered as esters from the skin and fins towards the end of the larval period. The most striking characteristic of the whole process is the emphasis on maintaining the colour pattern of the skin, and the importance of the carotenoids in this respect both to the spawning female and the developing larva.

The experiments in which up to 90 % of the yolk carotenoids were removed shortly after hatching support the view that these pigments are not essential for any vital physiological process during the larval period, or at least that the amount provided greatly exceeds any such requirement. The large amounts laid down in the ova provide the embryo with sufficient lutein and astacene to develop the colour pattern characteristic of the species during the larval period, before the young fish has commenced to seek its own food, a provision which may confer an important selective advantage. Removal of the carotenoids merely inhibits development of the colour pattern, but does not otherwise appear to affect the larva.

The distribution of β-carotene during the reproductive cycle differs from that of lutein and astacene. As shown previously (Steven, 1948), it is found in considerable amounts only in the livers of adult trout, and is not one of the pigments of the chromatophores. It is present in ripening oocytes and in freshly laid ova in smaller concentrations than lutein and astacene, but was not found in the bodies of embryos at any stage of development. It seems likely that it functions solely as a precursor of vitamin A, and is converted and utilized in the latter form by the embryo. This was the view of MacWalter & Drummond (1933), who claimed that the carotenoid concentration falls by about half during larval development, while the vitamin A increases. They were unaware at that time, however, that the ova of trout contain more than one type of carotenoid, which they considered to be similar to but not identical with β-carotene. Their estimates of the carotenoid content of ova were based upon measurements of the absorption of petroleum extracts at 480 mμ. Since, however, the absorption maxima of all three carotenoid fractions lie close to this wave-length, one would not expect a decrease of the β-carotene content to be easily detectable in the presence of much larger amounts of lutein and astacene, neither of which have ever been shown to act as precursor substances of vitamin A. This point clearly requires reinvestigation with the more sensitive methods now available.

SUMMARY

1. The free lutein and astacene in the muscles may contribute about half the carotenoid found in the ova of spawning female trout. The esterified forms of these pigments in the chromatophores of the skin are not, however, depleted.

2. Lutein and astacene are transferred without apparent loss from the yolk of the egg to the body of the embryo, principally during the later part of the larval period. The rate of pigment transfer appears to be related to the growth rate of the posterior end of the embryo, and corresponds closely with the visible development of xanthophores and erythrophores.

3. Removal by operation of about 90 % of the yolk carotenoids results in larvae which are slightly smaller than normal and with very few pale chromatophores, but with no other apparent defect.

4. Lutein and astacene in the yolk are the free hydroxy-carotenoids, but are esterified in the embryo. About two-thirds of the pigment of the embryo was found to be in the skin and fins at metamorphosis.

5. β-Carotene, which is present in freshly spawned ova, was not detected in the embryo at any stage of development.

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VASCULAR PATTERNS OF THE MAMMALIAN TESTIS AND THEIR FUNCTIONAL SIGNIFICANCE

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(With Plates 9 and 10 and Two Text-figures)

Even a superficial examination of the testes of various animals, for example rat and rabbit, is sufficient to make obvious that there are fundamental differences in their vascular supply and that the vessels display remarkable complexity both in their venous and arterial course. That morphological differences may exist between different animals has long been realized; thus de Graff (1677) clearly depicts the contrasting patterns of dog and dormouse. Nevertheless, comparative studies of these patterns have been meagre and their functional significance has not been elucidated.

In this communication is presented first a description of these testicular vascular patterns in a number of animals, drawing attention especially to the course and relations of the vessels within the spermatic cord. In a second section evidence is offered in support of the suggestion that the morphology of the blood vessels in the cord is connected with the temperature regulating mechanism of the testis—specifically, as a determining factor of the abdomino-testicular temperature 'gradient'.

I. ANATOMICAL

The blood supply of the mammalian testis has already been compared in forty-nine different species (Harrison, 1949), and therefore only those features of particular importance for this investigation will be described. These are concerned principally with the vessels in their course to the testis. The vascular patterns were investigated by the methods of arteriography and micro-arteriography (Barclay, 1947) after the injection of radio-opaque media into the testicular artery, and the relation of testicular veins to the artery observed in histological transverse sections at the site of convoluting of the artery. Diameter of the testicular artery was measured both on radiographs and histologically.

Dog. The testicular artery in the cord, when about $1\frac{1}{2}$ in. from the superior pole of the testis, forms a bundle of loosely packed irregular convolutions, as shown in Pl. 9, fig. 1. When the artery reaches the superior pole of the testis it straightens out, traverses the tunica albuginea obliquely, and passes down the posterior border of the testis to curve around the inferior pole of the organ and pass up its anterior surface, where it gives off its branches to the testis. The veins of the testis converge towards its posterior border to form the pampiniform plexus. These veins then pass up the

cord, surrounding the convolutions of the testicular artery as an anastomosing network. The looseness of the arterial convolutions in the cord of the dog as compared, for example, with those in the bull, was realized by Bimar (1888). The artery in the cord is 0·8 mm. in diameter, and forms twenty-five to thirty loosely arranged loops before reaching the testis. In a transverse section of the cord, the majority of the testicular veins are seen to lie around the testicular artery, but are separated from it slightly by connective tissue.

Goat (Pl. 9, fig. 2). Gutzschebauch (1935–6) appears to have been the only person to examine the vascular supply of the testis in this animal. The testicular artery on approaching the testis convolutes much more markedly than in the dog, in order to form a bundle of tightly packed convolutions. On reaching the superior pole of the testis the artery passes down the posterior border, convoluting only slightly, and divides into three to four branches which curve round the inferior pole, still convoluting, to reach all faces of the testis, from where the terminal arteries pass into the testis. The veins collect to form a pampiniform plexus in the cord closely surrounding the artery, which is about 0·9 mm. in diameter and forms over fifty loops in the cord closely packed together so as to produce a helicine formation.

Ram. The vascular pattern in the testis of this animal is almost exactly the same as in the goat, except that the testicular artery is wider in calibre (1·3 mm.) and forms more tightly packed convolutions in the cord. The artery is also more convoluted, forming about eighty wide spirals near the testis, as seen in Pl. 9, fig. 3, and is intimately related to the veins of the pampiniform plexus which have thin walls and anastomose profusely around the artery (Pl. 10, fig. 1).

Mouse (Pl. 9, fig. 4). The artery in passing to the testis is only slightly undulating, forming about seven 'half-loops', and shows the least convolution of any mammal, other than man, used in this investigation. The testicular vessels in the mouse, as in many other mammals, do not form a well-defined cord and are not in the same connective tissue sheath as the vas, so that it is difficult to comprehend a 'spermatic cord' in these animals. The artery passes down the posterior border, around the inferior pole and up the anterior border without showing convolutions. The artery has also the smallest diameter (0·1 mm.) of any testicular artery so far examined, and is completely surrounded by testicular veins without the intervention of connective tissue, as shown in Pl. 10, fig. 2.

Rat. There is marked coiling of the testicular artery in its course to the testis as shown by Pl. 9, fig. 5. On passing down the posterior border of the testis the artery narrows, but again widens on the anterior surface forming undulations before eventually giving off any branch to the testis. The artery before reaching the testis is about 0·2 mm. in diameter and forms approximately thirty closely packed loops in a mature animal. The veins of the pampiniform plexus are few, of small diameter and do not have such a close relationship to the testicular artery (Pl. 10, fig. 3) as in most of the other animals used in this investigation.

Rabbit. In passing to the testis the artery, which is 0·3 mm. in diameter, shows about thirty loops (shown in Pl. 9, fig. 6) which are packed almost as tightly as in the rat; on reaching the posterior border of the testis it constricts and then proceeds to

wind round the testis twice or even three times before supplying it, the single artery usually dividing into two after its first turn round the gland. The artery is almost completely surrounded by testicular veins, but separated from them by connective tissue, as seen in Pl. 10, fig. 4.

Guinea-pig. The artery convolutes only slightly in its course to the testis to form about ten complete loops, as can be seen from Pl. 10, fig. 7. After passing down the posterior border and around the inferior pole the artery gives off three or four collaterals which ramify over the anterior surface of the testis and send in branches to supply it. The artery is 0·2 mm. in diameter in an adult animal, and is intimately related to the veins of the pampiniform plexus, which surround it on all sides in direct contact with it (Pl. 10, fig. 5).

Cat (Pl. 9, fig. 8). The testicular artery, which is about 0·6 mm. in diameter, shows even fewer (about five) and less tightly packed convolutions in the cord than in the guinea-pig. On curving round the inferior pole the artery then passes almost up the middle of the anterior border giving off several branches on either side, similar to the arrangement in the dog. As seen in transverse sections (Pl. 10, fig. 6), the main artery is surrounded by comparatively discrete veins in close contact with it.

Table 1

Animal	Degree of convolution of the testicular artery No. of loops	Length on X-ray (cm.)	Diameter of the testicular artery (mm.)	Abdomino-testicular temp. diff. (° C.)
Ram	80	81·3	1·3	7·2
Goat	50	45·7	0·9	5·1
Rat	30	8·1	0·2	8·4
Rabbit	30	7·1	0·3	6·3
Dog	25–30	8·9	0·8	2·5
Rhesus	20	17·8	0·4	2·0
Guinea-pig	10	4·6	0·2	4·1
Cat	5	3·8	0·6	—
Mouse	7 (half loops)	1·0	0·1	8·4
Man	Straight	5·1	0·9	< 2·2 (Badenoch, 1945)

Rhesus monkey. The degree of convoluting of the artery is very similar to that in dog. The artery forms about twenty complete loops and then convolutes down the posterior border of the testis and at the inferior pole begins to form a complicated network of convoluting vessels, shown in Pl. 9, fig. 9. The artery is about 0·4 mm. in diameter, and is seen to be cut several times in a transverse section (Pl. 10, fig. 7) owing to the vertical looping of the artery in its course to the testis. The testicular veins are intermingled with the loops of testicular artery, but not in marked contact with them, being everywhere separated by connective tissue.

Man. The testicular artery, which averages 0·9 mm. in diameter, is almost straight in the cord, at least in the European; it may form at the most a few slight undulations as it nears the testis. The artery is completely surrounded by only a proportion of the testicular veins, which have thick walls and are separated from the artery by connective tissue (Pl. 10, fig. 8). The vascular pattern has already been described (Harrison & Barclay, 1948).

In all these species the course of the artery on the surface of the testis is immediately on the deep surface of the tunica albuginea. From the convoluted part of the artery in the cord, or just above it, branches are given to all parts of the epididymis. In no case are any branches given off to the testis until the artery reaches the anterior surface of the gland.

In summary it is possible to contrast the vascular patterns in the above by arranging them in a diminishing order of degree of convolution (Table 1), as assessed by the number of 'loops' seen on the X-ray pictures. The length of artery as measured on the radiograph, corresponding to the length from abdominal wall to superior pole of the testis, and the diameter of the testicular artery, are also given.

II. FUNCTIONAL

From the above description it is clear that there are wide variations in different species in the calibre and the course of the testicular artery both in its testicular course proper and in its course to the testis. These differences are indeed so marked that, in combination with other factors such as size and shape of testis, presence or absence of fatty body, no two species have as yet been found to be exactly alike in a comparative study of this vascular pattern in mammals. The relationship of the testicular artery to the pampiniform venous plexus also shows divergences.

The functional significance of these remarkable vascular arrangements has not, as far as we are aware, been appraised. We put forward here the suggestion that it is related to the thermo-regulatory characteristics of the testis. The arguments in favour of this suggestion are based on evidence presented in the following sections.

(i) *The abdomino-testicular temperature gradient*

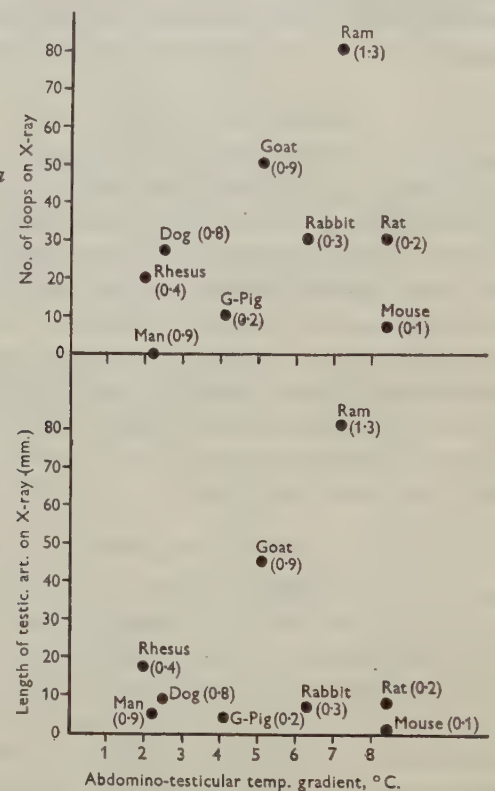
It is well recognized that a temperature gradient is maintained between the peritoneal and scrotal cavities, i.e. that testicular temperatures are lower than that of the abdominal cavity (Moore & Quick, 1924; Hammond & Asdell, 1927; Esser, 1932). We have confirmed the existence of this gradient as well as that between abdomen and the interior of the testis, in most of the animals dealt with above (Harrison & Weiner, 1948). These gradients vary as between different animals. In our series (Table 1) the abdomino-testicular gradient is smaller in rhesus monkey, dog, guinea-pig, or man (Harrenstein, 1928; Badenoch, 1945) than in goat, rabbit, rat, mouse or ram.

Some features of the vascular architecture would seem well adapted to bringing about and maintaining these gradients, and would also help to account to some extent for the observed species differences. The long intra-abdominal course combined with convolutions in the cord, and a small calibre are features of the testicular artery which would conceivably slow down the blood flow and give the arterial blood more time and surface for cooling. In conformity with these considerations is the finding that the less convoluted the testicular artery and the greater the calibre the smaller the gradient tends to be, as indicated in Text-fig. 1 *a*, the degree of convolution being expressed in the number of 'loops' given in Table 1 *a*. In Text-fig. 1 *a*, the results may be placed in two groups of greater and smaller arterial diameters and in

each of these groups there is a fair relationship of thermal gradient to convolutional complexity; the figure would support the *a priori* supposition that both degree of convolution and smallness of calibre are factors making for a greater thermal gradient. No doubt other factors are involved. The mouse, which has an almost unconvoluted artery has, in our series, a remarkably large temperature gradient; it will be noticed that it not only possesses the smallest calibre artery but also the smallest testis. The smaller the testis, the greater would be its cooling capacity in relation to its weight.

Text-fig. 1 *a*

Text-fig. 1 *b*

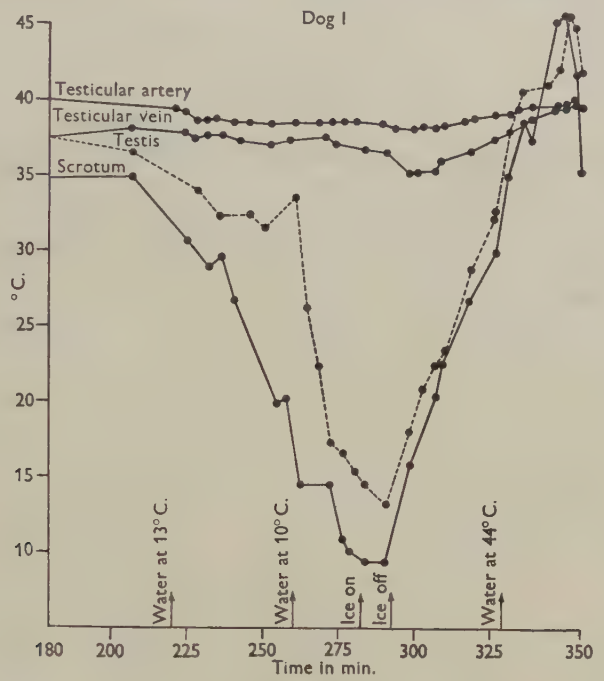


Text-fig. 1. *a*, Relation between number of convolutions, diameter of testicular artery in mm. (figures in brackets) and abdomino-testicular temperature differences (° C). *b*, Relation between length of testicular artery and abdomino-testicular temperature difference (° C). Diameter of testicular artery (mm.) is given in brackets.

We have attempted to estimate the length of artery as measured on the radiograph (Table 1) and to relate this to the gradient, since the relative lengths of the artery will depend on the size as well as the number of loops. The length so measured is obviously only an approximation; but one which would tend to underestimate the larger vessels due to the foreshortening on the radiograph, that is, those with larger loops. The relationship between length as seen on radiographs and gradient is shown in Text-fig. 1*b*. It suggests again that, in the arteries of larger calibre, the longer the artery the greater the gradient. The exceptions to this, presented by mouse, rabbit

and rat, emphasize again the factor of calibre, and also suggest that the number of convolutions apart from the length, determines the gradient. Other writers have given gradients for the rat which are smaller than ours (Moore & Quick, 1924; Esser, 1932); these figures would make our present results for the rat less anomalous.

Clearly factors other than those already mentioned, such as size, nature of coverings and effective contact with veins may be concerned. Previous writers (Crew, 1922) have directed attention in particular to the position and coverings of the testis (Esser, 1932; Phillips & McKenzie, 1934), but have neglected to consider the



Text-fig. 2*a*.

Text-fig. 2. *a–e*, Effect of heating and cooling the testis on intravascular temperatures and the temperature inside the scrotum. Abdominal and intratesticular temperatures are also shown in some cases.

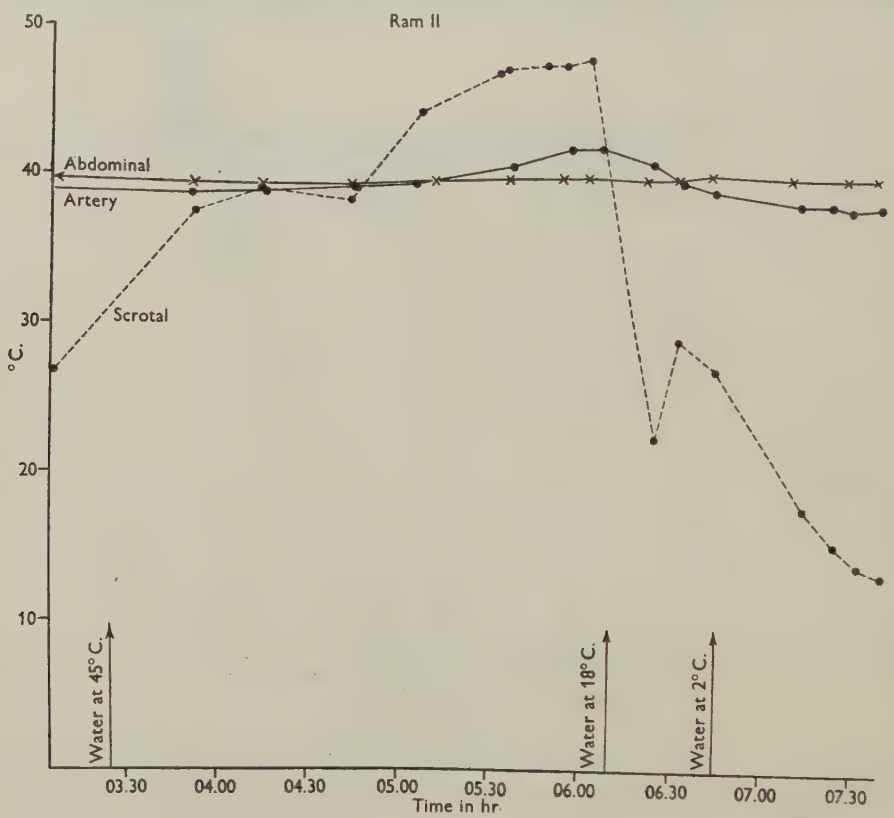
vascular factors. One may add that the relatively long passage of the testicular artery on the surface of the testis, described in the anatomical section above, would conceivably also contribute towards increasing the degree of cooling of the arterial blood before it reaches the interior of the organ. The goat, rhesus, ram, rat and rabbit show this feature rather more obviously than does the dog, man, cat, guinea-pig and mouse.

(ii) *The artery-vein relation in the cord*

Another anatomical feature which, in association with the features already mentioned, would appear to have a direct bearing on the mode of establishment of the abdomino-testicular temperature gradient is the relationship of the artery to the

pampiniform venous plexus. This venous plexus, as described in the anatomical section, is in close contact with the artery. In cross-section this relationship is shown very strikingly as we have seen. Manifestly a wide area for heat exchange is thus made available.

Since the venous blood leaving the testis would tend in any case to be cooler than the blood in the testicular artery leaving the abdomen, the close proximity of the two streams in the cord would serve to bring about precooling of the descending arterial blood flow; this would enhance the cooling features of tortuosity and small calibre



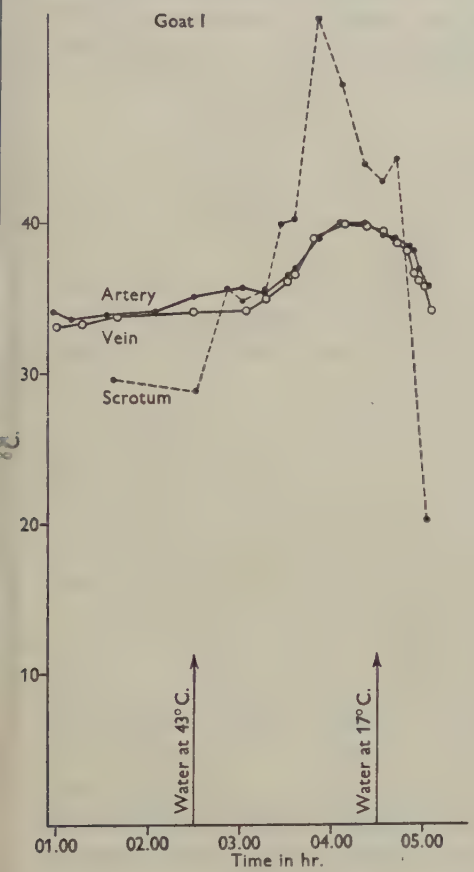
Text-fig. 2*b*.

already discussed. Such an arrangement would have the effect of bringing the *whole* testis to a temperature intermediate between that of the abdomen and the surface of the testis and cord. With 'precooling' of the artery there would be 'pre-heating' of the veins.

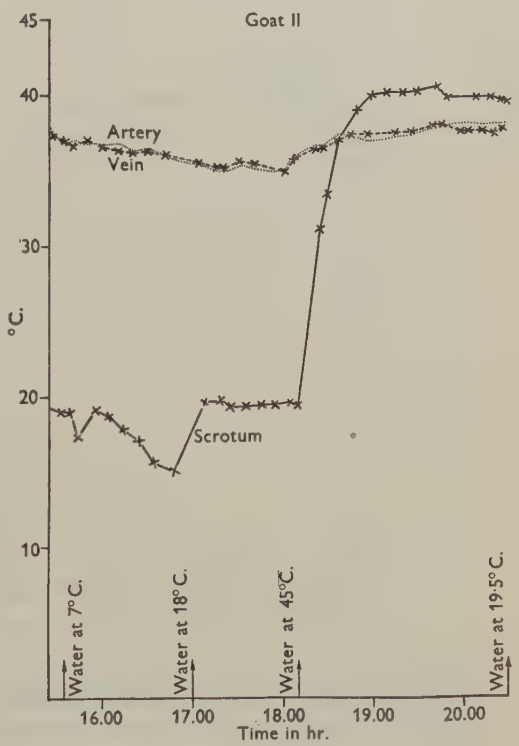
This mechanism is quite similar to that demonstrated by direct measurements of intravascular temperature by Bazett, Love, Newton, Eisenberg, Day & Forster (1948) (brachial, radial and common iliac arteries and superficial veins) and indirectly by calorimetry by Forster, Ferris & Day (1946) in the case of the blood flow in the human hand or foot. The latter found that considerable precooling of arterial blood

had to be assumed in order to account for the observed heat changes. Here there is 'precooling' of the arterial blood by venae comites. Cooling of the hand may bring about a lowering of temperature in the brachial artery quite far proximally (Bazett, *et al.* 1948).

In view of the anatomical characters of the testicular vascular pattern we should expect similar findings in the testes by measuring venous and arterial temperatures in the testicular vessels and observing the effects on these of cooling or heating the testis.



Text-fig. 2*c*.



Text-fig. 2*d*.

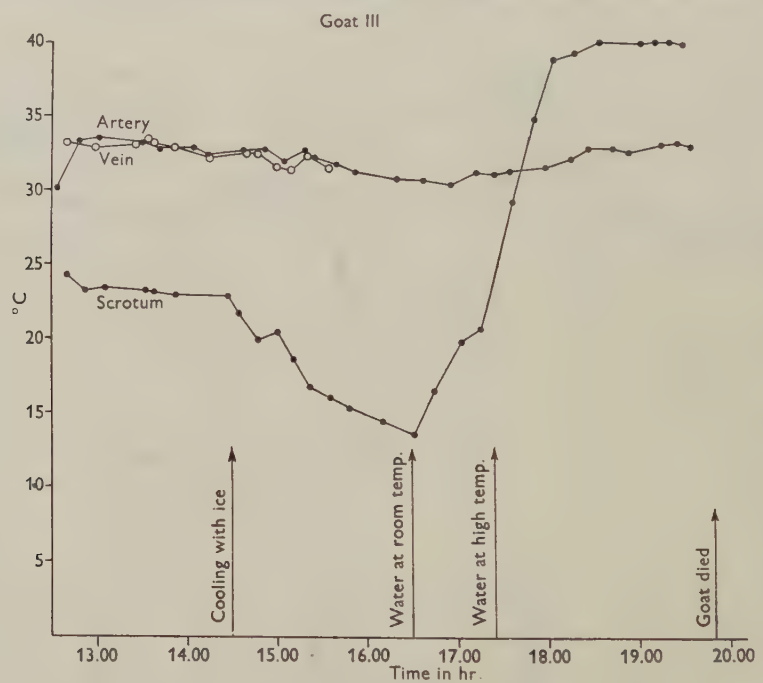
(iii) *Experiments on temperature changes*

Methods

Seven experiments using dogs (2), goats (3), and rams (2) were performed. Some of these proved unsuccessful in certain respects, particularly in the case of the dog where the testicular vessels are relatively narrow. Data relevant to the present investigation were obtained from all of these experiments; these are designated as Dog 1 or 2, Ram 1 or 2, Goat 1, 2 or 3.

The animal was anaesthetized by nembutal injected intraperitoneally (dogs and

goats), or intravenous chloral hydrate according to the method of Wright (1944) in the case of the rams, followed by ether. The spermatic cord was exposed as near to the abdominal wall as possible. Soldered copper-constantan (both s.w.g. 46) thermocouples were inserted into the testicular artery and vein. In all but experiment Dog 2, and the Goat experiments, the intravascular couples used were of the kind devised by Bazett *et al.* (1948) for their human experiments; in these the copper-constantan wires were secured in a fine plastic tube about 6–8 cm. long with the couple about $\frac{1}{4}$ cm. from the end. The plastic tube was inserted into the vessel and pushed along for a distance of 2 or 3 cm. and then moored by sewing it on to the nearby tissue and the margins of the incision. Similar couples were used to obtain



Text-fig. 2*e*.

the temperature within the testis, beneath the scrotum and in the abdominal cavity in the groin. The ram was found more suitable on account of the larger size of blood vessel; in Ram 2 the plastic tube slid out of the vein. Accordingly in the last three experiments (Goats 1, 2 and 3) the couple was fixed into a fine hypodermic needle (26 s.w.g.) cut down to just over 1 cm.; the whole needle slid into the vessel easily and became effectively anchored. Temperatures were taken for periods up to $1\frac{1}{2}$ hr. after closing the incision and then during and after heating and cooling of the testis. This was done by means of a copper coil round the testis through which water of required temperature from a thermostatic reservoir could be circulated. In experiment Goat 3 and probably Goat 2, the arterial and venous couples were fairly nearly at the same level in the cord; in the other experiments the arterial was definitely more proximal.

Results

(i) *Before heating or cooling the testis.* Where the couples were successfully inserted at roughly the same level (experiments on Goats 2 and 3) the arterial and venous temperatures are quite close; the arterial temperature is much the higher when the couple is more proximal than that in the venous plexus. These figures are given in Table 2, which also shows that the arterial temperature tends to be quite close to that of the abdominal cavity.

Table 2

	Abd. (° C.)	Testic. A. (° C.)	Testic. V. (° C.)
Dog 1	—	40·2	37·6
Ram 1	42·3	40·0	37·6
Ram 2	39·7	38·9	—
Goat 1	36·1	35·1	34·1
Goat 2	38·0	38·2	37·5
Goat 3	34·0	34·2	34·0

(ii) *Heating and cooling the testis* (see Text-fig. 2*a–e*). On heating the testis, intravascular temperature rises (Dog 1, Goats 2 and 3, Ram 2). Where the venous temperature is initially below that of the arterial (Dog 1) it rises more rapidly than that of the artery and may reach or surpass it (Dog 1, Goat 2). Thus it is possible for the thermal gradient to be reversed and for heat flow to be from vein to artery; and since arterial temperatures increase (Dog 1, Goats 2 and 3, Ram 2) and follow the venous (very closely when couples are at the same level—Goat 2) the results would be entirely in line with the occurrence of preheating of the arterial blood by the returning venous flow. We have also noted (Goat 2) that venous temperature during heating may exceed arterial for a time, then the lower temperatures become equal, the arterial again being higher and the sequence repeated.

It is manifestly also possible to precool the arterial blood in the cord proper (Dog 2, Goats 2 and 3, Ram 2). On cooling the testis venous temperature may fall rather more quickly than arterial (Dog 2) and arterial follows it (Goats 1 and 2).

Absolute proof of a passage of heat from one blood stream to the other would necessitate the calorimetric measurement of the testicular heat flux and its comparison with the flow of heat carried by the blood stream. This in turn would require an estimate of blood flow through the testis. In the absence of this information (which would necessitate considerable technical developmental work) we regard the data given above as in line with the anatomical evidence and in favour of an actual heat exchange mechanism between arteries and veins. We are fortified in this belief by the analogous results obtained in the hand and foot by the authors already mentioned.

DISCUSSION

The evidence presented above in support of the heat regulatory function of the vascular architecture is more or less indirect. Taking the anatomical and physiological findings, together with analogous results for other parts of the body, such as the hand and foot, we feel that our suggestion provides a reasonable explanation of the

function of these vascular arrangements. As has been stressed above, in the establishment of the temperature gradient between abdomen and testis other features such as the position and size of the testis and activity of the dartos muscle must be involved. Experiments demonstrating the regulatory role of the dartos muscle in countering changes in ambient temperature are on record (Phillips & McKenzie, 1934). If the vascular architecture plays a part, as we believe it does, then temperature regulation could conceivably be exercised through changes in blood flow as well. However, there are no data available on this point. Indeed we have not been able to find information as to the physiological stability or range of fluctuation of testicular temperature in the animals used in this investigation.

In warm conditions it seems likely that any effective cooling at the surface brought about by relaxation of the dartos muscle combined with increased heat loss by evaporation would be transmitted to the testis as a whole in so far as venous cooling could in turn be transferred to the artery through the vascular mechanism described in this paper. The effect of excessive cooling might, however, be enhanced by the operation of this mechanism. It may be that the mechanism is of greater importance in hot conditions since there is evidence that spermatogenesis is sensitive to a rise rather than to a fall in temperature (Phillips & McKenzie, 1934). Gronsky (1930) cooled rat scrota with ether until the scrotal temperature sank to 3° C. After 2–3 weeks there was atrophy of the germinal epithelium, spermatogonia, however, remaining, so that normal spermatogenesis had recovered within two months. Parkes (1945) has even frozen human semen in bulk at −196 or −79° C. and finds that a large proportion of the spermatozoa survives for long periods. Any cooling which the vascular mechanism may enhance may therefore not be organically deleterious, though in some species it may perhaps be concerned in the imposition of seasonal infertility.

On the other hand, Young (1929) estimates that a temperature of 45° C. or more has a marked effect on the fertilizing power of epididymal sperms from guinea-pigs, while a temperature of 46° C. for 30 min. (or 47° C. for 15 min.) applied to the testis in this animal caused an almost if not complete degeneration and desquamation of germinal epithelium in the seminiferous tubules (Young, 1927). In this connexion it is interesting to note that MacLeod & Hotchkiss (1941) have recorded a distinct fall in sperm count in healthy young men with normal total sperm counts following exposure to a temperature of 43° C. for more than half an hour in a fever cabinet. The counts reached a low level 25–55 days after treatment and were maintained for 15–50 days, after which the counts showed a relatively rapid rise.

As a corollary one may also ascribe to this vascular mechanism the role of protecting the testis from increases of body temperature generally. Thus in hyperpyrexia we might expect that the tortuosity of the artery and its precooling by returning veins, together with any other features improving heat dissipation, would serve to minimize the heating up from the body generally.

SUMMARY

The testicular artery in mammals convolutes to a variable extent before reaching the testis, and is closely surrounded by the veins of the pampiniform plexus. Evidence is presented suggesting that this vascular mechanism has a thermoregulatory function for the testis.

Species differences in the degree of convolution and calibre of the testicular artery and its relation to the veins of the pampiniform plexus would appear to account in some measure for observed differences in abdomino-testicular temperature gradient.

Experiments on dogs, rams, and goats also indicate that the close relation of the pampiniform plexus of veins to the testicular artery is well adapted to bringing about preheating or precooling of arterial blood flow to the testis.

We are indebted to Prof. H. C. Bazett for the method of soldering the thermocouples and gifts of plastic tubing, and for his interest in the initial stages of these experiments. We also wish to thank the late Dr A. E. Barclay for his help with the radiography in this investigation.

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EXPLANATION OF PLATES

Plate 9

Fig. 1. Radiograph of the testicular artery in the cord of a dog injected with 'Chlorbismol' (May and Baker). The convolutions of the artery are loosely arranged. Natural size.

Fig. 2. Radiograph of the testicular artery in the cord of a goat injected with 20% bismuth carbonate in normal saline. A slight burst has occurred at one point in the artery. Natural size.

Fig. 3. The pattern of the testicular artery in the cord of a ram revealed by radiography after the injection of 5% colloidal metallic bismuth and 'Wettal' into the testicular artery. Note the compactness of the convolutions. Natural size.

Fig. 4. The testicular artery of an albino mouse as shown by micro-arteriography after the injection of 5% colloidal metallic bismuth and 'Wettal' into the thoracic aorta. The testicular artery undulates before reaching the testis, forming about 7 'half-loops' and is seen to give off a large branch to the fatty body and caput epididymidis. (×4.)

Fig. 5. The vascular pattern of an albino rat testis as shown by micro-arteriography after the injection of thorotrast into the thoracic aorta. The convoluting of the testicular artery is clearly shown. The testicular veins can also be seen in the right lower corner of the figure congregating to a point on the postero-superior aspect of the testis. (×4.)

Fig. 6. Micro-arteriograph of the testicular artery in its course to the testis of a rabbit injected with 5% colloidal metallic bismuth and 'Wettal' through the thoracic aorta. Note the similarity in manner of convoluting of the artery with that in the rat. (×4.)

Fig. 7. Arteriograph of the vascular pattern in the guinea-pig testis, injected with 5% colloidal metallic bismuth and 'Cetavlon' into the thoracic aorta. The artery can be seen convoluting and then passing down the posterior border of the testis to curve around its inferior pole and then pass up the anterior surface where it gives off its terminal branches. Natural size.

Fig. 8. Radiograph of the testicular artery in the cat after injection of 'Chlorbismol' into the abdominal aorta. Note the few loops (about five) formed by the artery. Natural size.

Fig. 9. Radiograph showing the distribution of the testicular artery in a rhesus monkey after the injection of 5% colloidal metallic bismuth and 'Wettal' through the testicular artery. The artery can be seen to form complicated convolutions on the surface of the testis. Note the similarity in manner of convoluting of the artery with that in the dog. Natural size.

Plate 10

Fig. 1. Histological transverse section of the spermatic cord of a ram. The artery is seen cut several times and is surrounded by a profuse network of testicular veins. (×3.)

Fig. 2. Photomicrograph of a histological transverse section of the vascular bundle passing to the testis of a mouse. The artery is cut across one of its 'half-loops' and is completely surrounded by the veins of the pampiniform plexus. (×100.)

Fig. 3. Histological transverse section of the vascular bundle passing to the rat's testis. The artery has been cut through one of its convolutions and consequently appears irregular in shape in transverse section. The veins of the pampiniform plexus are few and are seen to bear no intimate relation to the artery. (×50.)

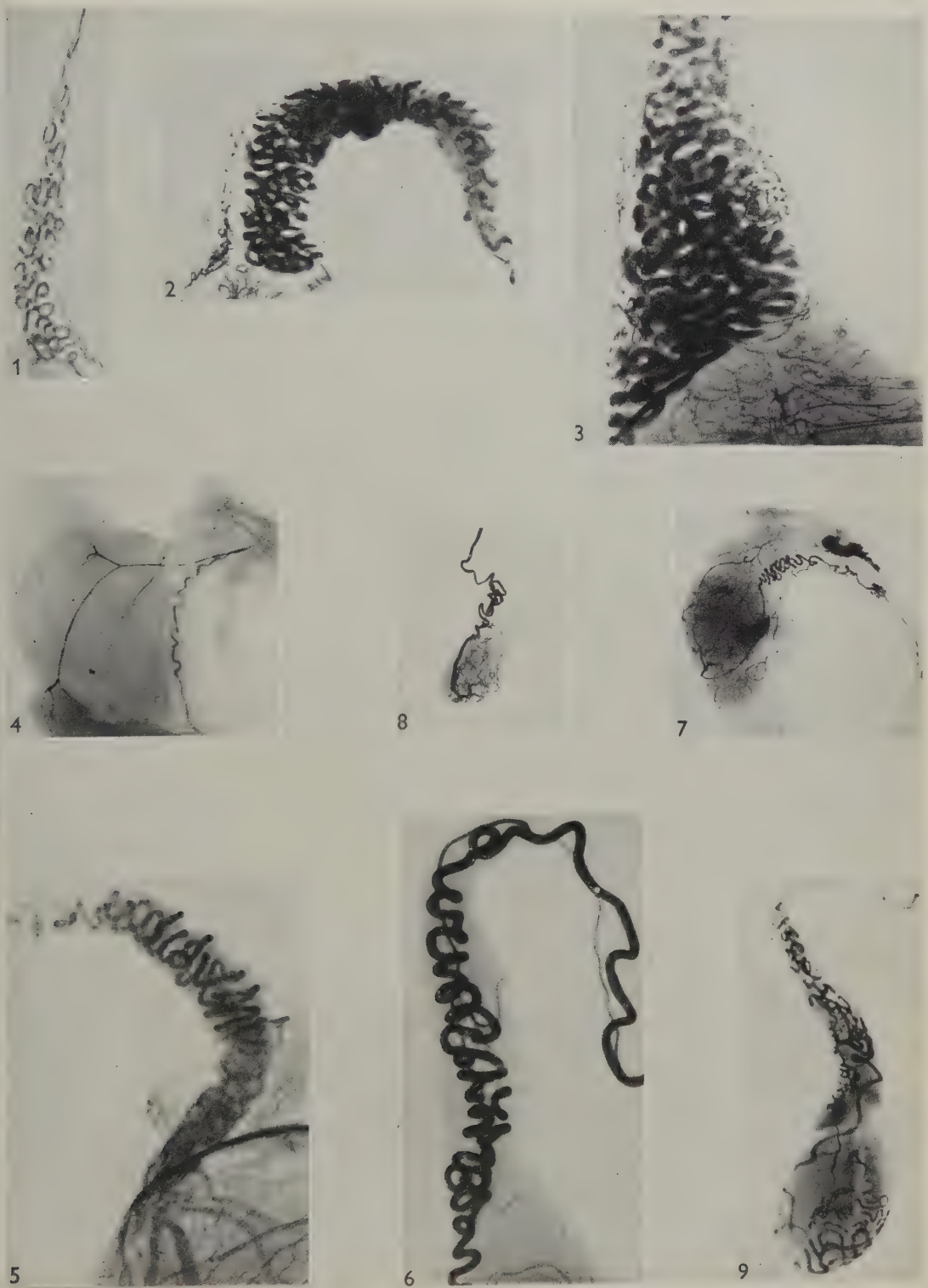
Fig. 4. The relation of the testicular artery and veins in a rabbit as shown by a histological transverse section. The main artery is seen to be elongated owing to section having been made across one of its loops, and is almost completely surrounded by testicular veins, but separated from them by connective tissue. (×50.)

Fig. 5. Photomicrograph of a histological transverse section of the testicular artery and veins in the guinea-pig. The artery is cut across its convolutions. Note the intimate relation of the testicular veins to the artery. (×50.)

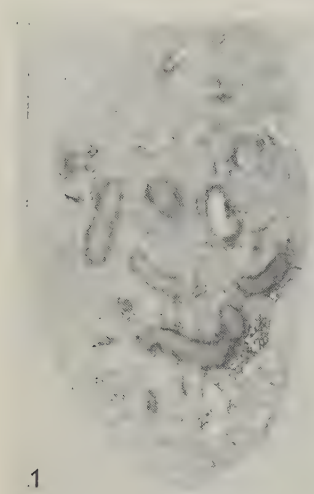
Fig. 6. Histological transverse section of the testicular artery and veins in a cat. The veins surround the main artery in close contact with it. The section was taken from a specimen in which the artery had been injected. (×20.)

Fig. 7. Transverse section of the cord in a rhesus monkey. The artery is cut several times, and the veins are separated from it by connective tissue. (×15.)

Fig. 8. Photomicrograph of a histological transverse section of a human spermatic cord, taken from an injected specimen. The artery is seen at about the middle of the figure. The vas is in the top right-hand corner. (×7.)



HARRISON AND WEINER—VASCULAR PATTERNS OF THE MAMMALIAN TESTIS AND THEIR FUNCTIONAL SIGNIFICANCE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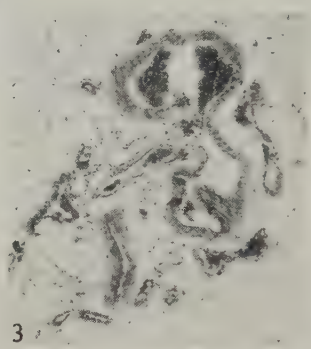
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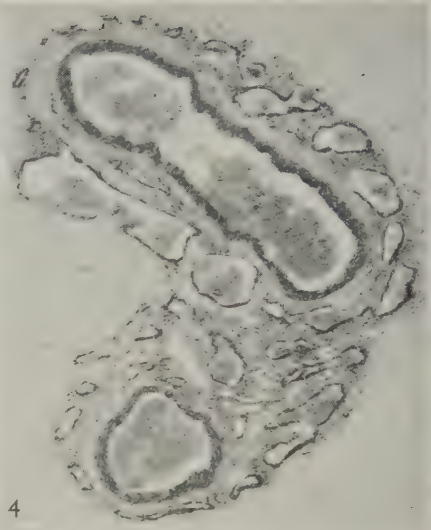
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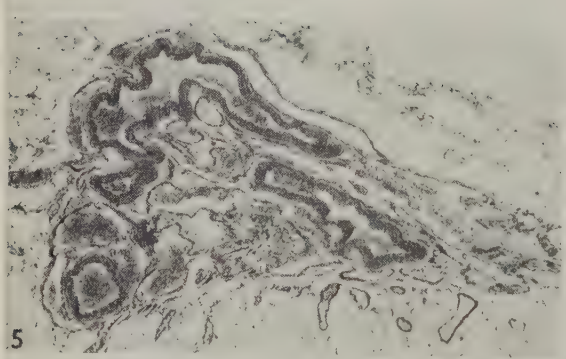
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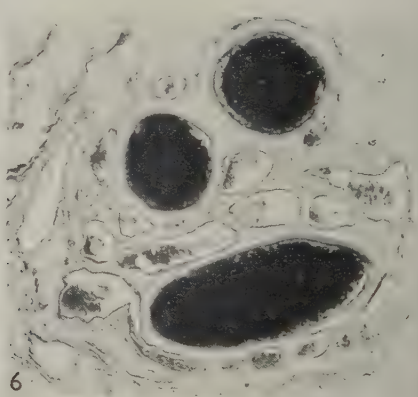
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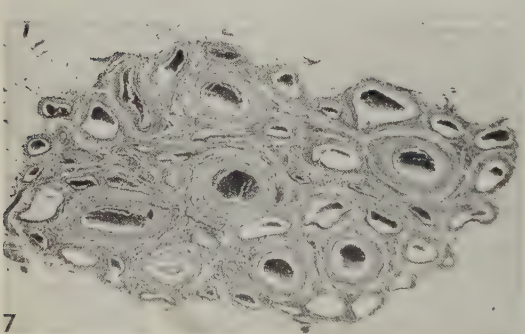
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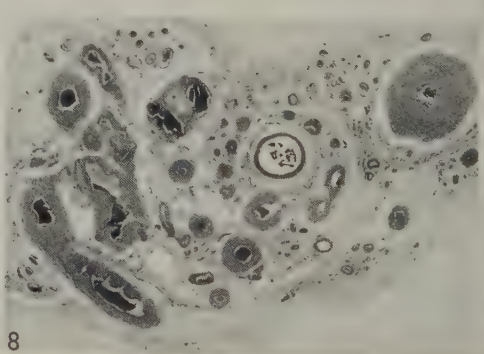
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HARRISON AND WEINER—VASCULAR PATTERNS OF THE MAMMALIAN TESTIS AND THEIR FUNCTIONAL SIGNIFICANCE

THE INFLUENCE OF AGE AND SEX ON 'REASONING'

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Genetically determined, or instinctive, behaviour arises from the selective effects of environment on the life history of a species, and at its first appearance in the life of an individual owes little or nothing to the previous experience of that individual. Most, if not all, organisms are also able to modify their behaviour through 'personal' experience. By their ability to learn, animals adapt themselves more intimately to their own particular environment. The new behaviour patterns set up are biologically advantageous to the individual. They arise out of, and are continuous with, the past history of the individual.

The essential mechanism of learning may be defined as the ability to combine two or more experiences which are spatially and temporally contiguous. The degree of learning depends, among other things, on repetition of such contiguity. It has also been suggested that some animals exhibit a type of mental process which is not genetically determined, and which is 'higher' than learning. Whereas learning requires that an animal should combine two or more experiences that have been presented in contiguity, higher mental processes, called *Einsicht* by Köhler (1927), reasoning by Maier (1929, 1932*a*, *b*, *c*), and symbolic processes by Morgan (1943), require that an animal should combine spontaneously two non-contiguous past experiences. Thus although the solution to a problem requiring 'reasoning'† is not discontinuous with the past history of the individual, since the experiences to be combined must have been present in that history, the particular temporal relationship between the experiences that is necessary to solve the problem has not been present in the individual's past history.

Evidence has been presented that the ability to solve the Maier three-table test of reasoning in rats (Maier, 1932*b*, *c*) is not the same process as the ability of a rat to learn (Campbell, 1935; Maier, 1932*b*), and this conclusion is confirmed by Vaughn's (1937) study of the intercorrelations between thirty-four variables in rat learning.

Since the Maier test is the only problem so far described which pre-pubertal and adolescent rats do not solve as well as adults, it was of interest to investigate, by the

* Dr Hanson did not receive the final proofs of this paper; he had previously expressed his indebtedness to Prof. S. Zuckerman for his advice and encouragement throughout this study.

† 'Reasoning' is used as a term of discourse throughout this article to indicate the mental processes by which rats solve the Maier three-table test described later in this paper. In so doing, no assumptions are made about the nature of the process, or about its relation to any human accomplishment. An extensive theoretical discussion of reasoning, and the spontaneous assembly of isolated segments of behaviour, is given by Hull (1935). Hull also discussed whether the ability to solve the problem described in this paper could be interpreted in terms of association theory, and proposed experiments that would test his conclusions. The results of some of these experiments had been reported by Wolfe & Spragg (1934).

use of this test, the role of sex hormones in the acquisition of the capacity to reason and thereby to discover the effect of gonadal maturation on post-natal neural maturation. It is well known that instinctive behaviour (e.g. sexual responses) can be induced by hormonal conditioning, and it has also recently been shown that performance of a learned response can be altered by hormonal conditioning (Douglas, Hanson & Zuckerman, 1948). The problems set in the present study were therefore, first, to confirm Maier's (1932*a*) conclusion that young rats do not solve the three-table problem as well as adults; and then to find how a premature puberty, induced and maintained from 22 days of age onwards by injection of a gonadotrophin, affects the capacity of pre-pubertal animals to solve the problem.

Since Maier's (1929, 1932*a*, *b*, *c*) early investigations on reasoning in rats, the three-table test has been used to investigate both the dependent and independent variables of the process of reasoning. One salient fact that is immediately obvious when one compares investigations on the effect of cerebral injury (Maier 1932*b*, *c*), diet (Wentworth, 1936), genetic stock (Loevinger, 1938), bromides (Hamilton & Harned, 1944), and glutamic acid (Hamilton & Maher, 1947), on reasoning, and on the relation of reasoning to learning (Campbell, 1935; Vaughn, 1937; Wolfe & Spragg, 1934), is that the success of animals has varied greatly in different investigations. Any satisfactory account of the three-table test should therefore indicate a basis for these wide variations. Suggestions that they are due to inherent differences in the reasoning capacity of animals of different genetic stock (e.g. Maier, 1935; Hamilton & Maher, 1947) only beg the question. The results of the present study lead to more specific answers to the question.

MATERIALS AND METHODS

Apparatus

The apparatus used in the three-table test for reasoning, which has been described in detail by Maier and other workers, consists essentially of three tables of different shapes. They are equidistant from and connected to a centre point by $1\frac{1}{2}$ in. wide and 3 ft. 6 in. long elevated wooden runways. Each table surface is of a different texture, and food placed on a table cannot be seen from the centre point, because a wooden shield is placed along the centrally directed edge of the table. Two such apparatuses, housed in adjacent rooms, were used in the present investigation. Since the results at no time differed significantly for comparable groups, the group results were pooled as if all the animals were performing on the same apparatus.

Subjects

One hundred albino rats (forty-eight males, fifty-two females) from eighteen litters were used. They were bred by the experimenter so that the exact age was known. After weaning, they were divided into groups of similar ages, as shown in Table 1. Each group contained the litter-mates of one sex from two or three litters, and comparable groups of litter-mates of each sex were used side by side on the test, so that the performance of the sexes could be compared. All except groups

VII and VIII, which are considered below, were fed in their cages until 7 days before the beginning of the test.

Vaginal smears were taken daily during the test period from group VI.

Table 1. *Composition of groups of experimental animals and sequence of experiments*

Series	Group no.	Age at test (days)	Cage no.	Sex	Injection	No. of animals		Dates of test
						In cage	In group	
1	I	30–50	H_1	Male	—	7	10	1. xii. 47–15. xii. 47
			H_2			3		
	II	30–50	J_1	Female	—	6	11	1. xii. 47–15. xii. 47
			J_2			5		
	III	50–70	B	Male	—	3	14	14. xi. 47–29. xi. 47
			D			9		7. xii. 47–21. xii. 47
			E			2		10. i. 48–24. i. 48
	IV	50–70	A	Female	—	5	19	14. xi. 47–29. xi. 47
			C			3		7. xii. 47–21. xii. 47
			M			11		10. i. 48–24. i. 48
	V	70–90	K	Male	—	6	12	14. i. 48–29. i. 48
			L			6		30. i. 48–12. ii. 48
	VI	70–90	N_1	Female	—	9	10	14. i. 48–28. i. 48
			N_2			1		30. i. 48–12. ii. 48
2	VII	30–50	O_1	Male	Gonadotrophin	6	12	13. ii. 48–28. ii. 48
			P_1	Male	Saline	6		
	VIII	30–50	O_2	Female	Gonadotrophin	6	12	13. ii. 48–28. ii. 48
			P_2	Female	Saline	6		

Hormonal treatment

Groups VII and VIII were taken from two large litters (containing fourteen and ten animals) which were born on the same day, and in which there were twelve males and twelve females. These litters were divided into males and females after weaning at 22 days of age. Six males (cage O_1) and six females (cage O_2) were then injected daily with 10 i.u. of pregnant-mare serum ('Antostab') in 0·1 c.c. saline. Their litter-mates (cages P_1, P_2) were injected with 0·1 c.c. saline only. The vaginae of the gonadotrophin-injected females opened 3–5 days after the first injection. The injections were continued daily until after the experiment was finished.

The methods of training and testing were similar to those used by Maier, Campbell, Loevinger and others.

Training

Groups of animals of the same age (± 2 days) were taken 7 days before the beginning of the test, and allowed to explore the apparatus for 1 hr. daily, after which each was given a large piece of bread soaked in milk in a separate cage, and was allowed to feed for 30 min. In their home cages they were allowed water *ad lib.* only. On one day each week neither training nor testing took place, and animals were fed in their home cages. 10 g. rat cake per animal was placed in each cage on this day. Within 6 days all animals had learned to run freely over the apparatus.

Testing

Fifteen tests were given, the first three of which are not included in the results. Testing was begun so that the tests on which scoring was based took place between 35 (±2) and 50, 55 (±2) and 70, and 75 (±2) and 90 days of age.

For the test, each animal was placed with a piece of bread soaked in milk on a table (food table) and allowed to nibble for 30 sec. The rat was then lifted from the table and placed directly on one of the other two tables (start table). If the animal returned directly to the table on which food was placed, the test was recorded as correctly solved. After feeding for 30 sec. on the food table, each animal was placed in a separate cage and allowed to feed for 30 min. In their home cages the animals were allowed water *ad lib.* only for the duration of the tests.

The two non-contiguous experiences thought by Maier to be combined in the solution of the problem of where the food lay were (*a*) knowledge of the apparatus acquired during training, and (*b*) knowledge of the table carrying food on the day of the test. Each day different start and food tables were used. Rotas were devised in which the six possible combinations of start and food tables were used in order. Each animal was on one of four different rotas, so that on any one day only a quarter of the animals was required to make the same response. This ensured that no olfactory trail was established.

Criterion of performance

A run was correct if the animal did not go more than half-way to the incorrect table. It was otherwise regarded as incorrect. The number of equivocal responses was very small.

RESULTS

The pooled results for each age group, in terms of right (+) and wrong (−) responses, are given in Table 2. The results are also scored in Maier's notation, so that comparison may be made with other workers' results. Maier expressed results as a percentage of right—wrong/total responses. According to his index the score that would result if the direction at the choice point were determined by chance only (i.e. 50 % correct responses), is marked as 0 %. Comparisons between groups of different

Table 2. *Pooled scores in the Maier test, in terms of right* (+) *and wrong* (−) *responses, of rats of comparable ages*

Groups	Age (days)	No. of animals	Score		Total trials	Success Maier index (%)
			+	−		
I and II	30–50	21	174	78	252	38·1
III and IV	50–70	33	274	122	396	38·5
V and VI	70–90	22	210	54	264	50·1
VII and VIII	30–50	24	249	39	288	72·9

age and sex were made by means of the χ^2 test, and results and comparisons are presented in Tables 3–6. In all comparisons there is only one degree of freedom.

The results show that in the first series of tests (groups I–VI) there is a gradient of acquisition of the ability to solve this problem (Table 2). Groups III and IV were not significantly more successful than groups I and II, whereas groups V and VI were significantly more so than either groups I and II or groups III and IV (Table 3). Comparison of the performance of males and females shows that between 70 and 90 days the males were significantly more successful than the females (Table 5). The females were also less successful than the males between 30 and 50 days and between 50 and 70 days of age. The differences at these ages are not statistically significant.

Table 3. *Comparisons of differences in performance of groups of rats of different ages*

Groups	Compared with groups	χ^2	P
I and II	III and IV	0·0015	>0·95
	V and VI	7·40	<0·01
	VII and VIII	23·97	<0·001
III and IV	V and VI	8·68	<0·01
	VII and VIII	27·68	<0·001
V and VI	VII and VIII	4·68	<0·05

Table 4. *Scores of each group of animals*

Group	Age (days)	Sex	No.	Score		Success Maier index (%)
				+	−	
I	30–50	Male	10	85	35	41·6
II	30–50	Female	11	89	43	34·8
III	50–70	Male	14	124	44	47·6
IV	50–70	Female	19	150	78	31·6
V	70–90	Male	12	120	24	66·7
VI	70–90	Female	10	90	30	50·0
VII E*	30–50	Male	6	63	9	75·0
VII C†	30–50	Male	6	64	8	77·8
VIII E*	30–50	Female	6	58	14	61·1
VIII C†	30–50	Female	6	64	8	77·8

* E = Experimental group. † C = Control group.

Table 5. *Comparisons of differences in performance of male and female rats in comparable age groups*

Males in group	Compared with females in group	χ^2	P	Pooled results	
				χ^2	P
I	II	0·34	0·50		
III	IV	2·93	0·05	5·559	<0·02
V	VI	9·589	0·01		
VII E	VIII E	1·34	0·20		
VII C	VIII C	0·07	0·70	0·74	>0·30

These results appear to confirm Maier's findings (1932*a*). The conclusion that young animals are unable to equal the reasoning ability of adults, drawn from the results of this test, was not, however, confirmed by the performance of the group receiving gonadotrophin and their control group. The pooled results from both sexes, and the results from both experimental and control groups independently, show that the performance of all groups in this later series was significantly more successful than that of any of the previous groups, and was as good as the most successful results reported in the literature (Tables 3, 4). There was no significant difference between the performance of experimental and control groups, or males and females, in groups VII and VIII (Tables 5, 6).

The results of all groups were also analysed to discover whether there was evidence of improvement in group performance during the course of the test. None was found in any group.

Table 6. *Comparison of performance of experimental and control animals in groups VII and VIII, together with comparison of difference in performance*

Groups		Scores		Total group score		Comparison of difference	
		+	−	+	−	χ^2	P
Experimental	VII	63	9	121	23	1·30	>0·20
VII E and VIII E	VIII	58	14				
Control	VII	64	8	128	16		
VII C and VIII C	VIII	64	8				

DISCUSSION

The wide variation in success of apparently comparable groups is thus similar to the variation found when the results of previous investigators are compared. The present results, however, are the work of one experimenter, and it is therefore possible to analyse what difference in the treatment of groups I–VI, on the one hand, and the control animals (which had been injected with saline only) in groups VII and VIII on the other, resulted in the greater ability of the latter to solve the problem.

There were two main differences in the treatment of the two sets of animals. Groups VII and VIII were handled extensively while receiving the injections of gonadotrophin and saline between 22 days of age and the end of the test period, and also, since they were tested last in the series, the experimenter had acquired greater experience and ability in conducting the tests. It is generally acknowledged that daily hypodermic injection of animals produces in time a marked adjustment in their somatic and autonomic responses to handling, and hence in the 'emotional' adjustment to other procedures. That 'emotional' adjustment may be a factor governing the success of rats on the three-table test has also been suggested in a postcript to Loevinger's analysis of reasoning in maze-bright and maze-dull rats (1938).

In the present experiment the rapidity with which the control animals in groups

VII and VIII became adjusted to the apparatus during training, and with which they responded during the tests, was in marked contrast to the slower time in adjustment and performance shown by earlier groups. No direct measurement of the former impression was made, but the time taken by groups VII and VIII to reach the food table after being placed on the start table was less than that taken by groups of animals tested earlier in the course of the experiment. Since the times for all animals were not recorded, these figures are not given.

Adequate 'emotional' adjustment is obviously not the only requirement for successful solution of the problem, but it is one that must be present before the factors in the situation upon which success depends can be utilized by the animal during the response. The results presented here show that 'emotional' adjustment may be the major variable on which success in solving the problem depends. The information that is given or implied in earlier reports confirms this conclusion, in so far as the most successful results recorded are those of Maier, who probably gained considerable experience in training animals while designing the apparatus. Many of his rats that had suffered cortical destruction were able to solve the problem with more success than intact animals at the hands of other workers. Similarly, Vaughn (1937) reports scores better than those that chance would determine, although his animals were required to solve a more difficult problem in which four tables were used. He also had handled these animals extensively in the course of earlier investigations. Wolfe & Spragg (1934), who present results showing evidence of improvement in group performance during the course of a test, suggest that previous training in similar situations may be necessary for successful performance. These authors are, however, considering specifically adjustment to the three-table test, and not adjustment to experimental procedures in general.

The present data also answers the question whether the better performance shown at 70–90 days of age compared to that at 50–70 days is entirely due to the fact that the older animals were tested later, when greater experience in the conduction of the test had been gained.

Although most of the tests on animals between 30 and 70 days of age were concluded before those on animals 70–90 days of age were begun, Table 2 shows that the animals in cages E and M, and K and N_1 (50–70 and 70–90 days of age respectively) were all tested between 10 and 29 January 1948. Since the handling of these animals was standardized, it is thought that the difference in age of the groups could be the only variable involved if a difference in their performance were found. A comparison between these groups shows that the group between 50 and 70 days of age performed less successfully than the group 70–90 days of age. The difference is highly significant (Table 7). Since, however, the sexes are unevenly balanced in these groups, and since it has been shown (Table 5) that females perform significantly less successfully than males, a comparison of the females only in each group is also given (Table 7). This shows that the older females were more successful, and that the probability that the difference is due to chance is between one in twenty and one in fifty. It may be noted that the female rats used were from a strain in which vaginal opening appeared most commonly between 45 and 55 days

of age. This indicates the possibility that the better performance of the older animals is due to their more adequate adjustment to the changes in external and internal environment that result from increased activity of sex hormones after puberty.

Table 7. *Comparison of groups in series* 1 *that were tested between* 10 *and* 29 *January* 1948

Cage no.	Age (days)	Sex	No.	Score		Total score		χ^2	P
				+	−	+	−		
E	50–70	Male	2	17	7				
M	50–70	Female	11	81	51	98	58	9·50	<0·01
K	70–90	Male	6	60	12				
N_1	70–90	Female	9	80	28	140	40		
M	50–70	Female	11	81	51	81	51	4·33	<0·05
N_1	70–90	Female	9	80	28	80	28		>0·02

It is therefore concluded that age may be a variable in the ability to perform this test, given that the 'emotional' adjustment of the animals is not complete. This suggests that the ease with which an animal adjusts emotionally to a situation is greater after 70 days of age than between 50 and 70 days of age, but that if adequate adjustment is made (and it can be made before 50 days of age), it is within the capacity of the pre-pubertal rat to solve the problem presented by the three-table test.

The cause of the significantly greater success of males than females also requires explanation. This sex difference is not observed in the results of groups VII and VIII, although the fact that the gonadotrophin-injected females do not appear to differ significantly from either the gonadotrophin-injected males or from the saline-injected females among their litter-mates may be attributable to the small size of the sample. The significant sex difference is not present either in the groups in series 1 of 30–50 and 50–70 days of age (groups I and II, and groups III and IV respectively), although the difference between the latter groups approaches significance. It may be noted that Maier (1935) found no difference between the performance of males and females, and that, as far as can be discovered, all other workers have used males only in this test.

The conclusion that can be drawn from Maier's results and those presented in this paper is therefore that when factors are present in the experimental situation that tend to prevent successful performance, the effects of these factors are greater on the post-pubertal female than the post-pubertal male. When, however, these factors are not sufficient to interfere markedly with performance, no sex difference is found. If it is possible to interpret 'reasoning' in terms of the effects of immediate environmental variations on the central nervous system, rather than in terms of central nervous system function divorced from immediate environmental control, the above conclusion may be restated in the following terms. If the connexion between stimulus and response is sufficiently strong, either because motivation is high or because no inhibiting factors are present, the factors tending to weaken the

response that are present only (or to a greater extent) in the post-pubertal female will be subthreshold. Such factors may, however, reach a threshold value if the strength of the connexion between stimulus and response is lessened.

Since Douglas *et al.* (1948) have reached a similar conclusion in an investigation on the effects of female sex hormone on learning, it seems possible that the processes involved in solving the three-table problem may be subject to the same modification as are these involved in learning, and that the difference between the sexes may be due to the effects of oestrogens on the female.

However, although the vaginal smears of group VI were taken daily during the test period, no significant difference could be found between percentage of success of animals in vaginal oestrus and of those in vaginal dioestrus. Thus, if there is a relationship between vaginal oestrus and lack of success in solving the problem, the relationship is not a direct one.

The results reported here thus suggest that the ability of rats to solve the problem presented by the Maier three-table test depends to a large extent on the adaptation of the animals to the situation, and that, given adequate adaptation, there are enough cues, either present at the moment of response or derived from the past experience of an animal, to enable a group of rats between 30 and 50 days of age to solve the problem in as high a proportion of trials as that recorded by adult rats in Maier's previous experiments.

If, however, adaptation to the experimental procedure is incomplete, both age and sex may be variables upon which the success of the response depends. Since it is possible for young females to solve the problem as successfully as adult males, the effects of age and sex must therefore be primarily on the adaptation of the animals, and not primarily on their capacity to 'reason'. Animals 50–70 days of age adapt less well to the apparatus than do animals 70–90 days of age, and post-pubertal females adapt less well than post-pubertal males. It is not certain what causes the physiological variations that underlie each of these differences, but it is possible that both are due to the effects of sex hormones on the nervous system.

SUMMARY

The effect of age, sex, and a prematurely induced puberty on the solving of Maier's three-table test by rats has been investigated.

It is found that rats 70–90 days of age are able to solve the problem more successfully than rats 30–70 days of age, unless the animals are fully adjusted to the experimental procedure. When fully adjusted, rats 30–50 days of age can solve the problem more successfully than animals 50–90 days of age, and just as successfully as the adults previously investigated by Maier.

Post-pubertal females have been found to be less successful in problem solving than post-pubertal males. The cause of this difference appears to lie in difference in the adjustment of animals to experimental procedure, and not primarily to differences in 'reasoning' ability.

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